Female reproductive health

Species- and ligand-specific endometrial differences to hormones and aryl hydrocarbon receptor activation

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Species- and ligand-specific endometrial differences to hormones and aryl hydrocarbon receptor activation

Vrouwelijke reproductieve gezondheid

Soort- en ligand-specifieke endometriale verschillen door hormonen en activatie van de aryl hydrocarbon receptor

(met een samenvatting in het Nederlands)

Proefschrift

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geboren op 17 februari 1990 te Apeldoorn

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Prof. dr. M.B.M. van Duursen

Content

Chapter 1

Introduction

General introduction

Over 10% of women in their reproductive age is affected by diseases of the female reproductive system such as endometriosis, endometrial cancer, and infertility [1–3]. As the prevalence of these diseases is increasing, there is emerging evidence linking them to the exposure to environmental contaminants [4–7]. However, the hormonal interplay between the brain, ovaries and uterus makes this system challenging to study. That is partly why good experimental testing strategies addressing the effects of environmental contaminants on female reproductive health are largely lacking.

In this Chapter, general concepts of the female human and rat reproductive cycles are introduced. Additionally, a mechanistic background as to how environmental contaminants may induce endometrial diseases is provided, with a focus on the role of the aryl hydrocarbon receptor (AHR). Finally, an overview of novel developments of models regarding endometrial health is provided.

1. Human reproductive cycle

1.1. HPG-axis

The female reproductive system is controlled by the hypothalamus and pituitary in the brain, and the ovaries in the pelvic region (HPG-axis). In this integrated system, the HPG-axis regulates the production of many hormones and relies heavily on multiple feedback loops between the individual endocrine glands. In short, gonadotropin-releasing hormone (GnRH) is excreted by the hypothalamus and exerts its action on the anterior pituitary to produce and release gonadotropins. These hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), regulate gonadal function like ovarian oocyte maturation, ovulation and transformation of the follicle into the ovarian corpus luteum. These hormones also stimulate the ovarian production of the sex hormones estradiol (E2) and progesterone (P4), which are essential in the regulation of the menstrual cycle (Figure 1) [8].

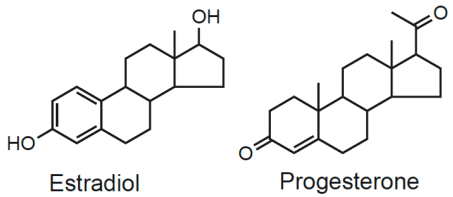

Figure 1. Structure formulas of sex steroids estradiol and progesterone. From [8].

1.2. Menstrual cycle

The menstrual cycle is unique to humans and primates and its interval varies from 20 to 35 days, with an average length of 28 days. In the first phase of the menstrual cycle, FSH stimulates the biosynthesis of estrogen from cholesterol in the granulosa cells of the follicle in the ovary. E2 acts on the endometrium, the inner lining of the uterus. As E2 levels increase, a negative feedback to the brain stimulates the release of LH and inhibits the production of FSH. Under the increasing levels of LH, also called LH surge or LH peak, at the midpoint of the menstrual cycle, an oocyte is released by the dominant follicle. This initiates the second part of the menstrual cycle, the secretory phase. Here, LH stimulates luteinization of the remaining follicular tissue in the ovary. This results in the formation of a corpus luteum or the yellow body. The corpus luteum then biosynthesizes and releases P4. As E2, P4 also acts on the endometrial cells to prepare the endometrium for the possible implantation of a fertilized oocyte. When conception fails to occur, the corpus luteum degrades and the production of P4 ceases. This drop in P4 level triggers the shedding of the endometrial lining, also known as menses or menstruation. Without the inhibitory feedback of the sex hormones to the brain, the hypothalamus again releases GnRH and the cycle begins anew (Figure 2) [8–10].

1.2.1. Proliferative and secretory phases in endometrium

The first part of the menstrual cycle is referred to as the proliferative phase, or the E2-dominant phase. Here, high E2 levels stimulate the endometrial cells to proliferate (Figure 3). During this phase, the endometrium thickens from several millimeters to approximately 12-14 millimeters under the influence of E2. Cellular division rates are high in the stroma, the lower layer of the endometrium, but particularly in the epithelium, the upper layer of the endometrium. Other growth factors such as epidermal growth factor and insulin-like growth factors produced by the epithelial and stromal cells are also involved in the autocrine or paracrine proliferation of the endometrial cells [11–13].

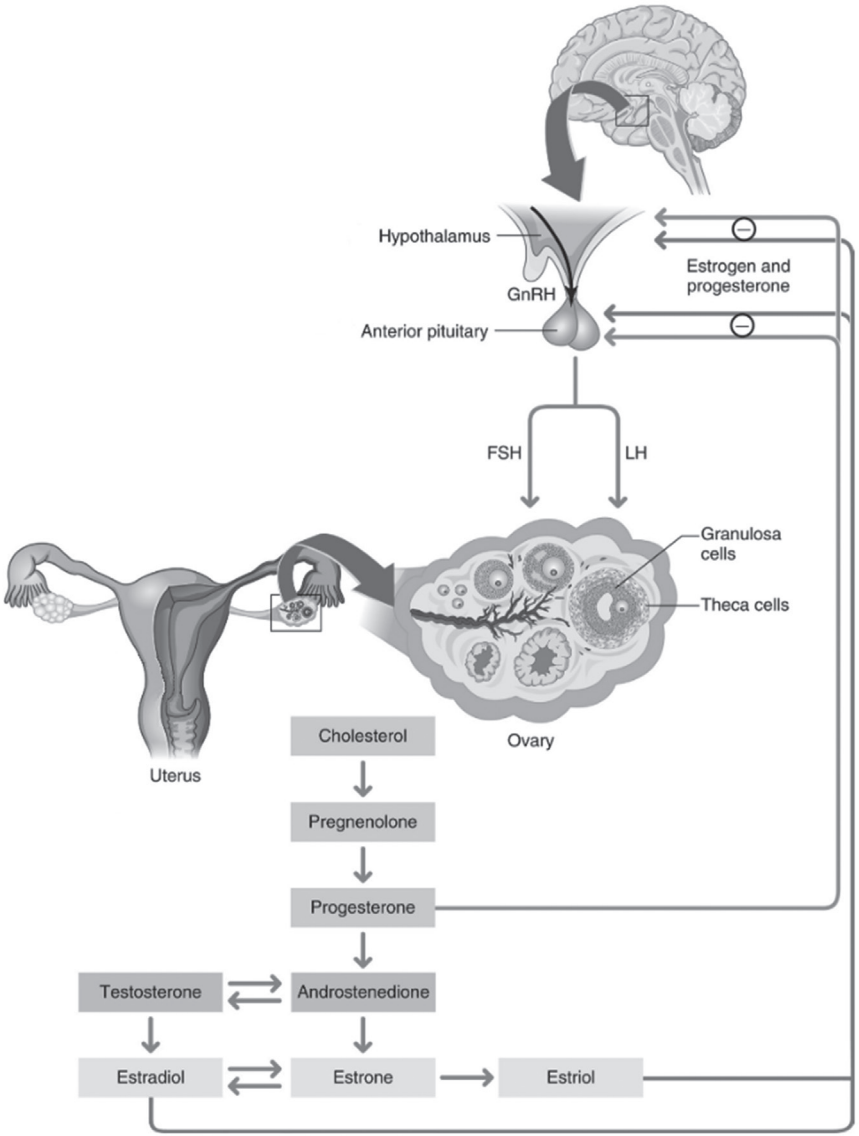

Figure 2. Overview of the female HPG-axis feedback loop. GnRH: gonadotropin releasing hormone; FSH: follicle stimulating hormone; LH: luteinizing hormone. Modified from [9].

During the secretory phase, or P4-dominant phase, of the menstrual cycle, the E2-primed endometrium comes under the control of P4 produced by the corpus luteum (Figure 3). As E2 levels decline and P4 levels increase, the proliferative behavior of the endometrial cells declines and instead shows secretory activity to prepare the endometrium for implantation of a fertilized oocyte. This process of cellular differentiation of endometrial cells is called decidualization. Approximately two days after the rise in P4 levels, the endometrial stromal cells start to produce and secrete prostaglandins (PG; PGE2). Both PGs and P4 enhance the proliferation of endothelial vascular filaments to stimulate formation of spiral or coiled arterioles [14, 15]. In addition, the endometrial glands grow and secrete large amounts of carbohydrates that can be used by the fertilized ovum as an energy source [16]. When conception does not occur, the corpus luteum degenerates and the declining levels of progesterone stimulate endometrial breakdown. This is initiated by enzymes such as matrix metalloproteinases (MMPs), which are induced by the declining levels of progesterone. MMPs are involved in the breakdown of transmembrane proteins and other components of the extra cellular matrix (ECM). Due to the falling P4 levels, the endometrium undergoes apoptosis, the vascular basement membranes break down, blood vessels are constricted and desquamation occurs, which result in the menstruation [16].

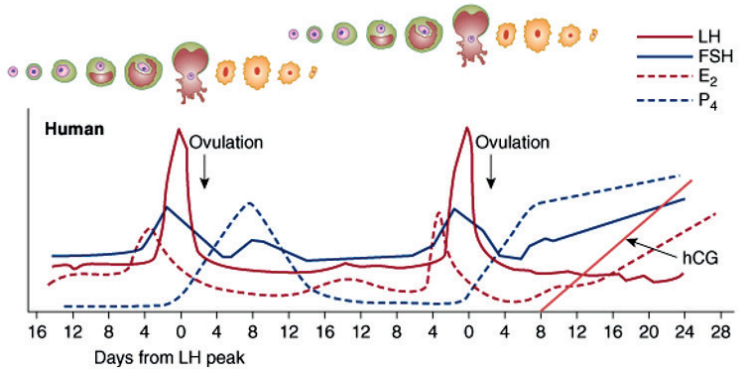

Figure 3. Overview of hormone levels during the proliferative and secretory phases of the (endometrial) menstrual cycle. LH: luteinizing hormone; FSH: follicle stimulating hormone; E2: estradiol; P4: progesterone. From [10].

1.2.2. *Anti-proliferative E2 function of P4 in endometrium*

E2 levels decline in the secretory phase of the reproductive cycle due to a low stimulation by FSH and thereby reduced production in the ovaries. But, the rising levels of P4 also contribute to the decline of E2-induced effects as it counteracts the function of E2. P4 can inhibit E2-induced endometrial proliferation via several mechanisms [14, 17, 18]. It can inhibit DNA synthesis in endometrial cells, as well as the expression of PCNA, Cyclin D1 and Cyclin A, which are essential components required for cell cycle activity [19]. Progesterone and the progesterone receptor (PR)-mediated action can also reduce the expression of the estrogen receptor (ER) [14, 20], as well as increase the metabolism of estrogen by inducing estrogen metabolizing enzyme catechol-O-methyl transferase [21]. As the target cell sensitivity to E2-signaling depends on the ER, this signaling can be impaired by the P4 action on the ER status of the endometrial cells. Additionally, P4 induces synthesis of the enzymes estrogen sulfotransferase and 17β-hydroxysteroid oxidoreductase (HSD) that inactivate E2. Estrogen sulfotransferase converts E2 to its inactive sulfoconjugate and 17β-HSD converts intracellular E2 to estrone (E1) [22, 23]. E1 has a much lower affinity to the ER compared to E2 and consequently has a lower estrogenic potential [24]. Because the action of hormones is determined by the balance between the expression of hormone receptors and steroid-activating or inactivating enzymes, together, this results in an inactivation of E2 signaling during the P4-dominant phase of the reproductive cycle [25].

1.2.3. *Hormone metabolism*

Steroids are mainly inactivated via hepatic metabolism which results in their excretion in urine. Extrahepatically, endometrial effects of steroids can be strongly affected by their bioavailability and local metabolism [26]. In fact, the ratio between E2/E1 in serum and local endometrial tissue shows marked differences [27]. This local metabolism of estrogen is partly regulated by enzymes which are under the control of P4 as mentioned previously.

In addition to the interconversion between weak or inactive and potent hormones via HSDs and sulfotransferases, estrogen metabolism can also be mediated by cytochrome p450 (CYP) 1A1 and CYP1B1 enzymes [28, 29]. Especially in hormone-dependent tissues, E2 and E1 are hydroxylated to catechol estrogens (CEs) 2-OH-estradiol and 4-OH-estradiol by CYP1A1 and CYP1B1, respectively [30]. CYP1A1/1B1-mediated estrogen metabolism yields CEs that can undergo methylation by catechol-o-methyl transferases (COMTs)

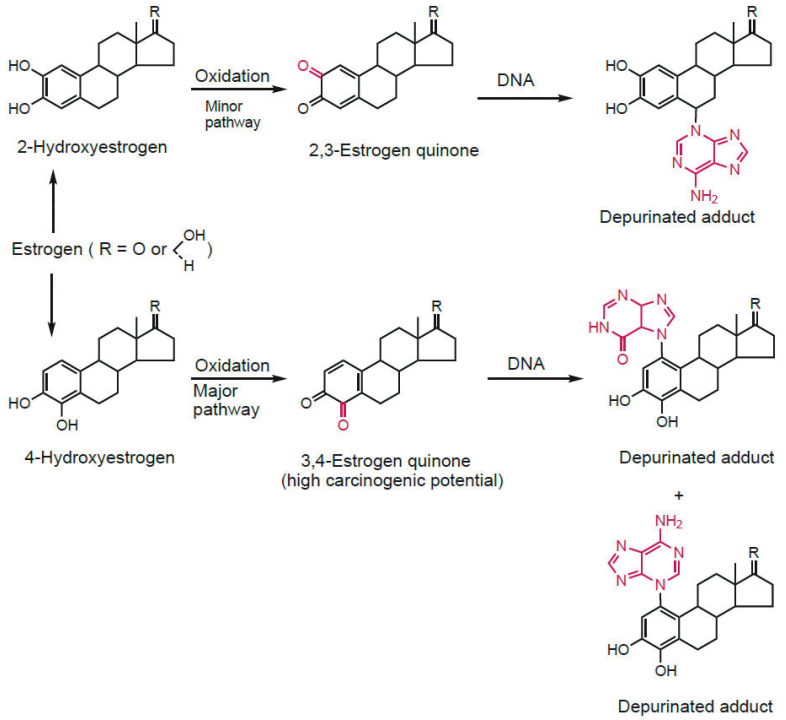

Figure 4. Overview of the metabolic pathways involving estrogens leading to DNA damage through depurination. From [8].

to inactivate them. If this process is incomplete, the CEs can be oxidized and yield reactive quinones thereby forming potentially harmful reactive oxygen species (ROS). Quinones of predominantly CYP1A1-derived 2-hydroxyestrogens (2-OHE1 or 2-OHE2) can form stable DNA adducts that remain in the DNA unless repaired. Quinones of the CYP1B1-derived 4-hydroxyestrogens (4-OHE1 of 4-OHE2) can however form depurinating DNA adducts, which results in the release of a nucleic base (Figure 4) [31]. As depurinating adduct formation induces DNA damage, this is considered a potential tumor-initiating event in human cancers, including endometrial cancer [32–36]. The expression of estrogen metabolizing enzymes thus play a critical role in regulating local

steroid levels and hormone balance. But, the control of the local levels of estrogens and their metabolites can also have a crucial role in the pathology of the endometrium [37].

1.3. Pathological effects

A disruption of the hormonal balance can give rise to numerous pathologies. Hormonal imbalances can be caused by external factors such as stress, malnutrition, birth control medicines and chemicals, but also medical conditions such as a pituitary gland tumor can result in a dysregulation of estrogen and progesterone levels. As the endometrial tissue is very hormonally responsive, a tight control of the hormonal balance is required for normal endometrial function [38]. As such, estrogens and progesterone can also be used to treat a wide variety of menstrual-related disturbances, including the management of menopausal symptoms, heavy menstrual bleeding, ovarian fibroids etc. Yet, persistent hormonal imbalance can result in severe pathologies, such as endometrial cancer and endometriosis, from which some of the etiologies remain poorly understood.

Endometrial (adeno) carcinoma, or endometrial cancer, is the most common gynecologic malignancy in developing countries, with an estimated 3% of women diagnosed during their lifetime [2]. A prolonged exposure to estrogen and insufficient P4 action are considered a high risk for endometrial cancer. As estrogen stimulates the proliferation of endometrial cells, unopposed estrogen can result in uncontrolled cell growth. In addition to this, some E2 metabolites can initiate DNA damage, which is considered a tumor-initiating event as described previously. As P4 antagonizes the estrogen-dependent proliferation of endometrial tumor cells, it has been used therapeutically for endometrial cancer [19]. High doses of progestins have also been used to treat endometriosis. This is a disease where endometrial epithelial and stromal cells are displaced and grow outside of the uterus, like in the ovaries, the fallopian tubes and within the pelvic region [39, 40]. Endometriosis, with varying intensity, is present in up to approximately 10% of women of reproductive age, and as the ectopic endometrial tissue continues to invade, proliferate, and breaks down in response to hormones it is a very painful condition [40]. Therapies are limited because the exact etiology of endometriosis is not fully understood. It is suggested that endometrial tissue often does not sufficiently respond to P4 and continues to grow under the influence of E2 [38]. Moreover, endometrial lesions often express aromatase enzymes that synthesize E2. As a result, suppression of ovarian E2 production cannot control the disease and its symptoms [40]. Polycystic ovary syndrome (PCOS) and luteal phase deficiency (LPD) are female endocrine disorders that can result in infertility as a result of hormonal disbalance, but their etiology remains unclear [41, 42]. Both proliferative and secretory phases of the menstrual cycle are required to work in series to sustain a normal secretory endometrium and the subsequent implantation of a blastocyst.

As increasing numbers of females are suffering from uterine diseases like endometrial cancer, endometriosis and (sub)infertility, testing strategies addressing disease etiology, effects of environmental contaminants and possible treatment options are warranted.

2. Species differences in the reproductive cycles

Our knowledge on female reproductive health (including endometrial pathologies) and the effect of exogenous compounds is often based on data obtained from *in vivo* animal studies. While *in vivo* studies using primates are most representative to the human situation, the use of primate studies is heavily debated for ethical reasons. Therefore, scientists often refer to rats and mice as a golden standard when assessing the effect of exogenous compounds, like newly developed pharmaceuticals or environmental contaminants, on female reproductive health. However, the rat reproductive cycle differs significantly from the human situation in several aspects. For example, the rat uterus is shaped in two long uterine horns that extend towards the kidneys. Such a duplex uterus accommodates multiple embryos, in contrast to a single uterus with a single chamber for the development of generally one single embryo in humans [43, 44]. Next to this structural difference, there are other, more mechanistic differences as well that will be further described below.

2.1. Estrous cycle

The rat reproductive cycle is an estrous cycle, in contrast to the menstrual cycle in humans. The difference is that where humans menstruate if conception does not occur, rats do not shed their endometrial layer of the uterus during their cycle. Instead, the cells in the endometrial lining are resorbed by the uterine wall in the rat [44].

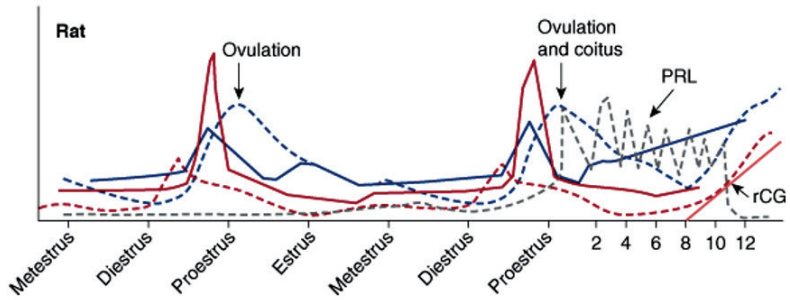

Figure 5. Overview of hormone levels during the rat estrous cycle. LH: luteinizing hormone; FSH: follicle stimulating hormone; E2: estradiol; P4: progesterone; PRL: prolactin. From [10].

The rat estrous cycle occurs every 4 to 5 days and consists of 4 distinct phases; the proestrus, estrus, metestrus, and diestrus [45]. Proestrus is the phase where the animal is "coming on heat", and lasts for 12 to 14 hours. During this phase, the LH surge induces ovulation [46]. Estrus is the phase where the female is willing to receive the male and ovulates, and typically lasts 25 to 27 hours. During estrus, extracellular fluid in the endometrium is increased due to high estrogen levels, which is often characterized by an increased endometrial weight [47, 48]. The estrus phase is followed by a short, 6 to 8 hours, so called recovery period; the metestrus. During this period, the changes in the reproductive tract subside. The metestrus is succeeded by diestrus, where the ovarian secretions of e.g. progesterone prepare the reproductive tract for receipt of the blastocyst for a period of 55 to 57 hours. If successful fertilization fails to occur, metestrus is followed by proestrus and the cycle begins anew [16].

Although the sequential stages of the rodent reproductive cycle correspond functionally to the proliferative and secretory phase of the human menstrual cycle, their control is differentially regulated. Particularly, the rat reproductive cycle does not display a distinct E2-dominant phase just before the LH peak like the human reproductive cycle (Figure 5). During proestrus, both estrogen and progesterone levels are elevated. After ovulation, estrogen levels fall whereas P4 levels remain high, similarly to the P4-dominant secretory phase in the human cycle. Additionally, the ovarian cycle is the same in both ovaries in rats, whereas the ovarian cycle alternates in humans [49].

2.2. Hormonal regulation

Estrogen and P4 are, similar to the human situation, produced in the rat ovaries. Their regulation however, differs in some aspects from the human situation. The estrous cycle is subject to change in response to environmental factors such as light and seasons [50]. GnRH and LH secretion are regulated by the feedback loop in the HPG-axis depending on E2 levels, but also by a neuroendocrine mechanism that depends on circadian signals [46, 51]. Even high carbohydrate or high protein diets can prolong the estrous cycle in rats [52]. Such factors are not known to influence the human reproductive cycle.

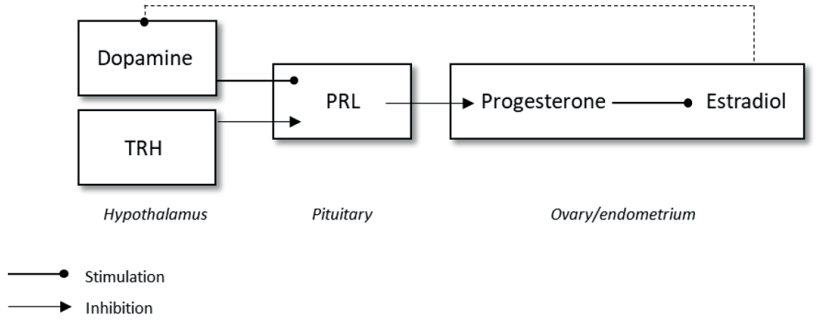

Figure 6. Schematic overview of the prolactin regulation in rats. PRL: prolactin, TRH: thyrotropin-releasing hormone.

There is also a difference in the function of the pituitary hormone prolactin. Rats exhibit a particularly sensitive feedback mechanism between estrogens and pituitary prolactin secretion, while in humans, endogenous estrogens only have a modest stimulatory effect on pituitary prolactin release [53, 54]. Rising estrogen levels drive the secretion of prolactin in rats by interacting with dopamine releasing systems in the hypothalamus (Figure 6) [55, 56]. Dopamine, secreted from the hypothalamus, inhibits prolactin release and gene transcription via the dopamine receptor D2 (D2R) on the pituitary [57]. On the other hand, hypothalamic Thyrotropin-Releasing Hormone (TRH) is a direct stimulus for prolactin release via the TRH receptor in pituitary cells in both rats and humans. This is important because prolactin is luteotropic in rats, in contrast to humans, i.e. it promotes the production of progesterone in the corpus luteum after ovulation and maintains gestation [53, 54].

There also is a dissimilarity in local estrogen metabolism regulation between the rat and human. In humans, important enzymes in this local metabolism are 17β-HSD and sulfotransferase as mentioned previously. In humans, their activity is mainly under the control of P4. Although 17β-HSD is present in the rat endometrium, its activity is highly stimulated by estrogen [58]. In addition, the presence of sulfotransferase cannot be detected in the rat uterus in contrast to humans [59]. Instead, estrogen is glucuronidated by UDP-glucuronosyl transferase (GT) in the rat uterus [23]. However, progesterone stimulates UDP-GT and thus the subsequent glucuronidation of E2. It appears that these estrogen-inactivating enzymes are regulated in an almost opposite way in rats [23].

Similar to the human situation is that E2 metabolism is also (partly) mediated by CYP1A1 and CYP1B1 in rats. E2 metabolites 2-OH-E2 and 4-OH-E2 can be measured hepatically as well as extrahepatically and their production is increased with increasing CYP1A1 and CYP1B1 expression in rats [60, 61]. Also, P4-mediated antagonism of E2/ER signaling is observed in rats [62].

3. Xenobiotics in endometrium

Despite the rising incidence of uterine diseases in humans [4, 6, 63], the etiology behind these diseases is still poorly understood but generally involve hormonal dysregulation in the reproductive organs. Increasing evidence shows that exposure to exogenous compounds, like environmental contaminants, chemicals or pharmaceuticals, may contribute to the development of reproductive diseases in various species, including humans [5, 7, 64]. For example, a role for dioxins and dioxin-like substances has been suggested in the pathogenesis of endometriosis in humans [65, 66]. Dioxins and other environmental contaminants such as bisphenol A (BPA) have also been associated with reduced couple fecundity (time to pregnancy) and PCOS in epidemiological studies [42, 67, 68].

The aryl hydrocarbon receptor (AHR) plays an important role in the effects caused by environmental contaminants, as it is involved in the detoxification and clearance of exogenous (toxic) substances from the body. But its aberrant activation has been linked to many disease states including endometrial cancer, reduced fertility and endometriosis [69].

3.1. General AHR

The AHR plays a key role in the detoxification processes in response to exogenous compounds (see also in paragraph 3.1.1), but the physiological function of the AHR in the absence of such compounds is unclear. The AHR appears vastly important in the early development of organs such as the immune, hepatic, cardiovascular and reproductive systems [70, 71]. Transgenic mouse and rat models have been developed where the AHR was suppressed or blocked, which confirmed that the AHR has a fundamental role in normal development and cell and organ physiology. Many of these older AHR-null mice were found to have a number of abnormal phenotypes including a peripheral immune system deficiency, heart hypertrophy, severe localized epidermal hyperplasia in the skin, hyperproliferation of blood vessels in the liver, calcifications in the uterus and several adenocarcinomas [72].

The AHR is a ligand-dependent/activated basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) containing transcription factor. When inactive, it resides in the cytosol where it is held stably in an open conformation by its association with a protein complex of co-chaperones AHR-interacting protein (AIP), p23 (phosphoprotein 23 kDa), and heat shock protein 90kDa (hsp90) [73]. Ligand binding to the AHR ligand binding domain (LBD) triggers conformational changes of the PAS-AHR structure which results in a dissociation of the cytoplasmic proteins and stimulates its translocation to the nucleus [74, 75]. The dimerization of the AHR with the Ah receptor nuclear translocator (ARNT) protein converses the AHR into its DNA binding form [73, 76]. The resulting ligand:AHR:ARNT complex can then bind to specific DNA binding sites referred to as xenobiotic response elements (XRE) or dioxin-like response elements (DRE), in the promotor regions upstream of AHR target genes (Figure 7) [77, 78]. Most studied is the AHR-activated gene battery involving CYP1A1 and CYP1B1 which are often used as a proxy for AHR activation.

3.1.1. CYP1A1/1B1

CYPs that are induced by ligand-mediated AHR activation are often involved in the metabolic breakdown and elimination of those ligands, a process referred to as biotransformation. CYPs are phase I oxidative enzymes that add polar groups to the parent compound to increase solubility preparing for excretion of said compounds. Sometimes, an AHR ligand can be metabolized into a more bioactive or reactive metabolite, referred to as bioactivation. Here, the metabolite, rather than the parent compound, can exert toxicity in a cell or organism.

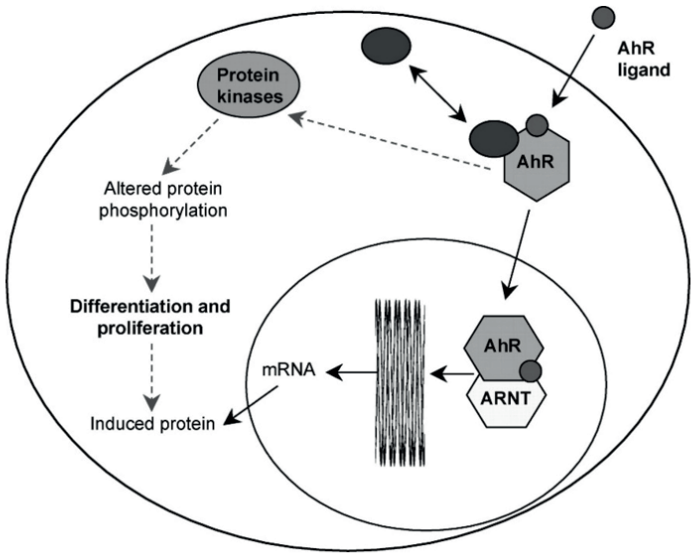

Figure 7. Schematic overview of aryl hydrocarbon receptor activation. From [69].

This can occur via several mechanisms, but CYPs are involved in many of the most common modifications of a parent compound. The functional effects of CYPs include oxidation or reduction of the parent compound, including aliphatic or aromatic hydroxylation, epoxidation, N-, O-, or S-dealkylation, sulfoxidation, desulfuration and oxidative dehalogenation [79, 80]. For example, the induced CYP system is directly related to the (geno)toxicity of PAHs like benzo-a-pyrene (BaP) [81]. CYP1A1, CYP1B1, and to a lesser extent CYP1A2 are responsible for the transformation of BaP to several mutagenic intermediates [80, 81]. Some of these metabolites have been identified as reactive compounds, like benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) [84]. Such reactive epoxides are able to interact with DNA, which results in the formation of DNA-adducts. Adduct formation may result in mutations and gene silencing, as covalent DNA binding may block the DNA repair system and inhibit transcription of DNA [85]. Such DNA adducts can be considered tumor-initiating events, similar to the depurinating DNA adducts formed by estrogen metabolites after CYP metabolism.

3.1.2. ER-AHR cross-talk

The AHR exhibits extensive cross-talk with the ER, which may partly explain its important role in (the development of) the reproductive system. Not only are CYP1A1 and CYP1B1 involved in the metabolism of E2 (as mentioned in 1.2.2.), there are various other mechanism via which AHR activation can affect E2 signaling [84, 85].

Similar to activation of the AHR, ARNT is involved as a coactivator for ER activation [88]. Competition for ARNT and subsequent reduced E2 signaling can be observed in cells that are exposed to AHR ligands. Additionally, squelching of other shared co-factors with steroid receptors has been reported which can lead to a repression of ER signaling [86]. AHR activation can also result in the inhibition of ER signaling by interfering with ER-DNA binding to specific estrogen responsive elements (EREs) that contain an overlapping XRE/DREs sequence. This may lead to inhibition of selected E2-target genes, stimulating ER proteasomal degradation, and/or other as yet unidentified mechanisms (Figure 8) [85, 87–90].

The AHR can function as a repressor of E2 signaling, however, the functional relationship between AHR and ER is highly complex. Its interaction with the ER appears to be dependent on the presence of respective ligands, the nature of the ligands and cell/tissue-specific co-factors involved in the activation of the receptors [87, 91–93]. As E2-signaling is crucial to the endometrial health and normal endometrial functioning, the responses to AHR ligands in the endometrium clearly should be taken into account when studying female reproductive health.

3.2. Ligand differences

The AHR was historically studied because of its role in mediating the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [96]. Exposure to high levels of TCDD can result in chloracne and immune toxicity in humans. However, the long-term effect of low levels of TCDD exposure in humans have been under evaluated [70]. Yet, it is clear that the binding of TCDD to the AHR and its consequent activation is the key event in TCDD toxicity.

TCDD is the most potent AHR ligand and was implicated in the development of endometriosis in rhesus monkeys [97] and in humans following accidental exposure to dioxin in Seveso, Italy [66]. However, conflicting data are published

in terms of a mouse model with surgically induced endometriosis where TCDD reduced the proliferation in endometriotic lesions that was induced by E2 [98]. Additionally, dioxin-type environmental compounds slightly increased the incidence of uterine carcinoma in a 2-year rat study *in vivo* [91, 97]. While the exact mechanism of TCDD toxicity on the endometrium is unclear, it is suggested that it results from inappropriate and sustained TCDD-mediated activation of the AHR [71, 84].

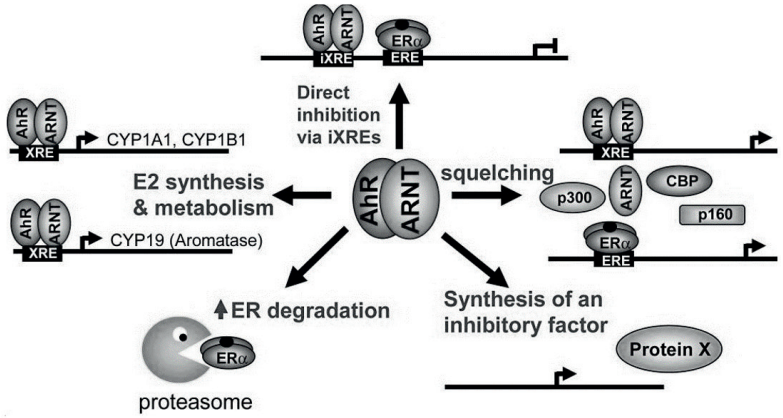

Figure 8. Overview of possible mechanisms between AHR and ER signaling. Modified from [90].

Next to TCDD, there are various other exogenous compounds that bind and activate the AHR, which can result in a wide range of biological and toxic effects across many species and tissues [77]. The best characterized and high-affinity AHR ligands are halogenated aromatic hydrocarbons like TCDD, dibenzofurans, biphenyls, various polycyclic aromatic hydrocarbons (PAH) and PAH-like chemicals. However, there are also naturally occurring plant hormones that are AHR ligands like resveratrol and quercetin, although these exhibit a lower AHR binding affinity [77, 78]. In addition, there are also some pharmaceuticals that have been identified as AHR ligands, such as Laquinimod, or its metabolite DELAQ, which is currently being evaluated for the treatment of Huntington's disease [100]. More recently, endogenous AHR ligands *e.g.* FICZ, kynurenin and kynurenic acid, as part of the L-tryphan pathway, have been identified AHR ligands [99, 100].

This large array of structurally diverse AHR ligands suggests that this receptor has a highly promiscuous LBD. Moreover, significant differences in ligand-specific AHR functionality, responses and LBD specificity have been reported across tissues, but also across species [73, 77, 101].

3.3. Species differences

Although the AHR seems to be evolutionary conserved between many species (e.g. humans, rodents, *Caenorhabditis elegans, Drosphila melanogaster* and *Danio rerio*) [104], ligand-binding does not always evoke a similar response. For example, *C. elegans* lacks the expression of the CYP1 family. Exposure to compounds like BaP that are metabolized to toxic intermediates by CYP1 enzymes therefore do not result in a (geno)toxic response in *C. elegans* [105]. Rodents however do express the CYP1 family enzymes, and activation of the AHR appears to induce similar canonical pathways as compared to humans [70].

The potency of similar AHR ligands like dioxins, furans and dioxin-like PCBs on the AHR have long been characterized using toxic equivalency factors (TEFs) [104, 105]. The relative potency of a compound to activate the AHR was compared to an index chemical, TCDD which is known as the most potent AHR ligand. For example, PCB-126 was found to be 10 times less potent than TCDD in a wide range of rodent *in vitro* and *in vivo* studies, and therefore was assigned a TEF of 0.1. However, it was suggested that TEFs can differ between rodents and humans. More recent studies showed that the TEF of PCB-126 in human cells is actually closer to 0.01 than 0.1 in rodent cells [106, 107]. This finding indicates that the relative potency of AHR ligands indeed differs between species.

Species-specific differences have also been identified with respect to AHR-ER cross-talk (as mentioned in 3.1.2.) [87, 110]. Rats may not require the same co-factors as humans do for ER dependent induction of E2-depending genes. Thus, squelching by the AHR may not have the same effect in different species and tissues depending on the type of co-activators available and/or required [86].

4. Current endometrial test models

It is clear that AHR activation can disrupt endometrial hormone signaling and hormonal balance. Although, at present, it remains challenging to study the effects of environmental contaminants on endometrial diseases by translating *in vivo* rodent toxicity studies to a human situation. Not only are there vast differences between the human and rodent reproductive cycles, it also appears that AHR activation may display strong ligand, tissue, and species differences. Yet, current regulatory testing strategies lack relevant predictive (*in vitro*) models for humans with respect to hormone disruption and female reproductive health [93].

4.1. Cell lines and primary cells

There are several human endometrial cell lines available that have been used to study the effects of chemicals on endometrial cells. Such cell lines are often derived from endometrial adenocarcinomas (such as ECC, Ishikawa, HEC1A or KLE cell lines), or endometrial primary cells that have been modified to "survive" in the laboratories (hTERT-EEC cells) [85, 91, 109–112]. It is however questionable to what extent these cell lines represent a "normal" healthy endometrial cell. Moreover, these *in vitro* models do not take into account the interplay between various endometrial cell types, metabolism, hormones and feedback mechanisms.

However, "normal" primary endometrial cells can however be isolated and harvested from a human uterus. Although these cells have limited survival time during *in vitro* experiments, they actually reflect a more accurate model to study the effects of chemicals on the endometrium. Nevertheless, it should be recognized that these primary endometrial cells do not necessarily reflect a healthy human endometrial microenvironment with interaction between epithelial and stromal cells, which is imperative for physiologically relevant behavior.

4.2. 3D models

Others have studied the effects of endometrial explants, i.e. small minced fragments of endometrial tissue, or isolated both primary epithelial and stromal cells from endometrial tissue. Such models allow researchers to study the effects of chemicals as well as the interplay between various endometrial cell types [113–117]. The effects of endometrial tumor cell lines were also studied in combination with healthy endometrial stromal cells, which created a tumor microenvironment [118–120].

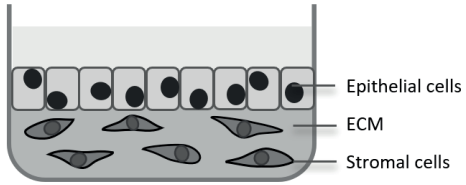

Figure 9. Schematic representation of the 3D endometrial model. ECM: extracellular matrix.

In this thesis, a 3D endometrium model using primary human epithelial and stromal cells was established based on the models of others (Figure 9) [113, 116, 117]. The endometrial cells were isolated from fresh uterine tissue donated by healthy premenopausal patients undergoing hysterectomy for non-malignant reasons. Endometrial epithelial and stromal cells were cultured separately and subsequently incorporated into a 3D model. This model consisted of 2 layers; a bottom layer where an extracellular matrix scaffold encloses the primary stromal cells and an upper layer of primary epithelial cells to allow polarization of the epithelial cells (Figure 9). Moreover, the layers were exposed to different hormonal exposure scenarios, mimicking the proliferative and secretory phases of the reproductive cycle. A similar 3D model was developed using rat primary endometrial cells.

5. Aim of this thesis

The aim of this thesis was to assess endometrial differences between human and rats as a platform to develop novel, human relevant test methods for female reproductive health. In addition, we aimed to identify species-specific differences between human and rat and tissue-specific differences upon AHR activation.

In Chapter 2 of this thesis we describe the development of a 3D endometrial co-culture model to assess the differences between primary human and rat endometrial cells in response to hormones and AHR activation. In Chapter 3,

the differences between the interplay with E2 signaling and AHR activation in primary human and rat endometrial epithelial cells were assessed. Another aspect of human-rat differences and hormonal response is evaluated in Chapter 4, where the effect of AHR activation on rat pituitary prolactin regulation was investigated. Chapter 5 and 6 address tissue-specific (and ligand-specific) differences after AHR activation between human endometrial and breast tumor cell lines. Chapter 7 summarizes the results described in Chapter 2-6 and provides a general discussion on the content of this thesis.

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Chapter 2

Primary endometrial 3D co-cultures: A comparison between human and rat endometrium

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Abstract

Human and rat reproductive systems differ significantly with respect to hormonal cyclicity and endometrial cell behavior. However, species-differences in endometrial cell responses upon hormonal stimulation and exposure to potentially toxic compounds are poorly characterized. In this study, human and rat endometrial hormonal responses were assessed *in vitro* using a 3D co-culture model of primary human and rat endometrial cells. The models were exposed to the aryl hydrocarbon receptor (AHR) ligands 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), laquinimod, and its AHR active metabolite DELAQ.

In both the human and rat endometrial models, estrogen receptor and progesterone receptor gene expression was modulated by the hormonal treatments, comparable to the *in vivo* situation. AHR gene expression in the human endometrial model did not change when exposed to hormones. In contrast, AHR expression decreased 2-fold in the rat model when exposed to predominantly progesterone, which resulted in a 2.8-fold attenuation of gene expression induction of cytochrome P450 1A1 (CYP1A1) by TCDD. TCDD and DELAQ, but not laquinimod, concentration-dependently induced CYP1A1 gene expression in both human and rat endometrial models. Interestingly, the relative degree of DELAQ to induce CYP1A1 was higher than that of TCDD in the human model, while it was lower in the rat model.

These data clearly show species-differences in response to hormones and AHR ligands between human and rat endometrial cells *in vitro*, which might greatly affect the applicability of the rat as translational model for human endometrial effects. This warrants further development of human relevant, endometrium-specific test methods for risk assessment purposes.

1. Introduction

Uterine diseases and disorders like endometriosis, uterine tumors and infertility are conditions that seriously harm female reproductive health, with each disease affecting about 10% of women in their lifetime [1-3]. Despite the rising incidence of uterine diseases [4, 5], the pathogenesis behind these diseases is still poorly understood but generally involve hormonal dysregulation in the reproductive organs. Increasing evidence shows that exposure to environmental contaminants may contribute to the development of reproductive diseases in various species, including humans [6–9]. For example, a role for dioxin-like substances has been suggested in the pathogenesis of endometriosis [10]. Dioxins and numerous other environmental contaminants have also been associated with reduced couple fecundity in epidemiological studies [11].

Exposure to dioxins, *e.g*. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), can disrupt estrogen signaling *via* activation of the aryl hydrocarbon receptor (AHR) and consequently cause reproductive dysfunction [12]. AHR activation can result in the inhibition of estrogen receptor (ER) signaling by interfering with ER-DNA binding to specific estrogen responsive elements (EREs) that contain an overlapping dioxin response element sequence. This may lead to inhibition of selected estradiol (E2)-target genes, stimulating ER proteasomal degradation, increasing estrogen metabolism by AHR-inducible cytochrome P450 (CYP) enzymes and/or other as yet unidentified mechanisms [13–17]. Consequently, compounds that activate the AHR may have the ability to inhibit estrogen signaling pathways. Indeed, TCDD has been shown to inhibit ER-mediated chemical-activated luciferase reporter gene expression (ER-CALUX) in human breast cancer cell lines *in vitro* [17, 18]. Although estrogenic regulation between breast and endometrial tissue sometimes differs, inhibition of E2 signaling *via* AHR activation has been associated with anti-cancer properties of TCDD as it can inhibit estrogen-induced proliferation in endometrial adenocarcinoma cell lines (ECC1 and HEC-1A) and in female breast cancer MCF-7 cells *in vitro* and in *in vivo* mice studies [19–22].

Studying the effects of chemical agents like TCDD on the female reproductive organs remains challenging because of the continuously changing hormone levels, and the interplay between various cell types in the endometrium throughout the menstrual cycle. The first phase of the menstrual cycle, the proliferative phase, is reflected by the thickening of uterine endometrium under

the influence of E2. After ovulation, the endometrium is maintained under the influence of progesterone (P4) which is secreted by the corpus luteum. When fertilization does not occur, P4 levels drop in the secretory phase and the breakdown of the endometrial wall is promoted by an increase in *e.g*. matrix metalloproteases (MMPs) that degrade the extracellular matrix [23].

Currently, toxicological research predominantly uses *in vivo* rodent models to assess possible adverse effects of compounds on female reproductive health. Yet, the rodent estrous cycle differs significantly from the menstrual cycle, which is unique to humans and primates [24]. Not only do rodents not menstruate, the regulatory patterns and specific roles of certain hormones differ as well. For example, pituitary hormone prolactin stimulates the production of progesterone in rats, but not in humans [25]. In addition to these differences in the reproductive cycle, rodents differ also in sensitivity to certain classes of chemical compounds compared to humans. For example, rat liver cells show a higher sensitivity for cytochrome P450 1A1 (CYP1A1) induction than human liver cells in response to TCDD [26]. Thus, it is likely that present testing strategies in toxicological risk assessment do not adequately predict human endometrial effects. However, a clear comparison between responses of human and rat endometrial cells has never been made. Commercially available human (tumorigenic) endometrial cell lines like HEC1A, KLE and Ishikawa are sometimes used to study endometrial effects *in vitro*. These studies are typically performed with mono-cultured cells that lack the interaction with surrounding extracellular matrix and stromal fibroblasts, which is crucial for epithelial cell behavior.

In this study, primary healthy human and rat endometrial cells were obtained and their response to hormones was investigated *in vitro*. For that, a three dimensional (3D) *in vitro* endometrial co-culture model was established using primary healthy human and rat endometrial epithelial and stromal cells, based on previously described models [27–30]. The endometrial cells were exposed to relevant hormonal concentrations to mimic the various stages of the reproductive cycle (E2 dominant and P4 dominant). Changes in gene expression of nuclear hormone receptors ERα, progesterone receptor (PR) and the AHR were determined, because of their important role in endocrine processes. Considering the ability of the pharmaceutical laquinimod to activate the AHR [31], the effects on AHR gene expression and activation of CYP1A1 by laquinimod, and its metabolite DELAQ, as well as TCDD were assessed in these human and rat *in vitro* endometrial models.

2. Material and Methods

2.1. Human endometrial cell isolation

Human endometrial tissue was obtained from healthy premenopausal women undergoing hysterectomy for non-malignant conditions at the St. Antonius Hospital (Nieuwegein, the Netherlands). The study was approved by the hospital's Medical Ethical committee (Study number Z15.038) and written informed consent for the use of the endometrial tissue was obtained from all patients. None of the donors were using hormonal medication prior to or at time of surgery. Immediately after surgery, the endometrium was placed in HBSS (Thermo Fisher Scientific, the Netherlands) + 1% 100 U/ml penicillin/ 100 µg/ml streptomycin (p/s; Thermo Fisher Scientific) buffer and kept at 4 °C until further processing. The protocol for isolating and separating the endometrial cells was based on a combination of previous work of several laboratories [27–30]. Upon arrival in the lab, the endometrial tissue was incubated in 0.25% trypsin (Thermo Fisher Scientific) for 1 h at 37 °C in a shaking water bath. Then, the endometrial tissue was minced into smaller pieces and incubated in 0.05% DNAse type I (Sigma-Aldrich, the Netherlands), 0.1% collagenase type II (Sigma-Aldrich) and 0.1% trypsin in HBSS for 1 h and 15 min in a 37 °C shaking water bath. The suspension was flushed over a 40 µm nylon mesh cell strainer (Corning, the Netherlands) to allow stromal cells to pass through the filter, whereas it retained the epithelial cells. All cellular fractions were placed separately into culture flasks. After 1 h and 20 min, the supernatant of the cultures was transferred to a new culture flask to further separate stromal and epithelial cells by selective adherence. Human endometrial cell fractions were cultured in RMPI-1640 medium (Thermo Fisher Scientific) + 1% p/s + 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and maintained in an incubator at 37°C in 5% CO_2.

2.2 Rat endometrial cell isolation

Animal experiments were carried out in accordance with the Dutch law for animal welfare, and protocols were approved by the Ethical Committee of Animal Research of Utrecht University (AVD108002015135) and the Institutional Animal Care Committee of Utrecht University (WP800-15-135-01-002). For the rat endometrial cell isolations, 15 female Sprague-Dawley rats aged 7 weeks (Envigo, the Netherlands) were euthanized by using O_2/CO_2 asphyxiation upon arrival in the animal facility. The uteri were dissected and placed in cold HBSS + 1% p/s buffer. Uterine horns were subsequently separated

and slit longitudinally. The horns were incubated in 0.25% trypsin for 1 h in a shaking water bath at 37 °C, the supernatant was seeded in a culture flask and transferred to a new flask after 1 h. The uterine horns were subsequently incubated in HBSS containing 0.01% DNAse type I, 0.05% collagenase type II, and 0.08% trypsin. After 1 h and 15 min in a shaking water bath at 37 °C, supernatant was collected and the cells were flushed over 40 µm nylon mesh cell strainer to separate epithelial and stromal cells. Both fractions were collected and seeded in separate culture flasks. After 1 h, the supernatant was transferred to new culture flasks. Rat endometrial cells were cultured similarly to the human endometrial cells.

2.3. Experimental setup

Approximately 5 days after isolation, culture media of the endometrial cultures was replaced with culture media containing charcoal-stripped FBS (Hyclone, GE Health Care, the Netherlands). Two days later, cells were detached from the culture flasks with Accutase (Thermo Fisher Scientific). Stromal cells were plated at a density of 70.000 cells/well in 120 µl extra cellular matrix (ECM, Sigma-Aldrich) gel:medium (1:1) in a 24-well plate. After 30 min at 37 °C, 1 ml of 70.000 patient-paired epithelial cells/mL was added to each well on top of the ECM containing stromal cells. Both stromal and epithelial cells were seeded in medium that contained hormones to mimic the different phases of the reproductive cycle. For human cells this included a vehicle control (0.2% EtOH), an E2 dominant situation (100 nM E2 (Sigma-Aldrich) and 1 nM P4 (Sigma-Aldrich)), and a P4 dominant situation (1 nM E2 and 100 nM P4) [29, 30]. For the rat cells, a vehicle control (0.2% EtOH) and a P4 dominant treatment (1 nM E2 and 100 nM P4) were used. No E2 dominant phase was included, as the rat estrous cycle lacks an apparent E2 dominant phase. After 3 days, half of the medium was removed and replaced with fresh medium with corresponding hormone and compound concentrations (Figure 1). Experiments were performed in triplicate. After 2 days of exposure, culture media was collected and RNA Instapure (Eurogentec, Belgium) was added to the cells. Samples were stored at -80 °C for further analysis.

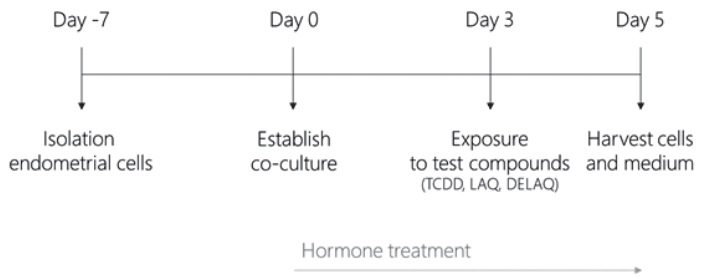

Figure 1. Timeline of the experimental setup in this study.

2.4. Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR)

The RNA of the endometrial cultures was isolated using the phenol–chloroform extraction method and RNA Instapure to lysate the cells as described previously [32]. The 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 iScript cDNA Synthesis Kit (BioRad, the Netherlands), 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 using the CFX96 cycler (BioRad). A PCR mastermix was made containing IQ SYBR Green Supermix (BioRad), a forward and a reverse primer, after which cDNA of the sample was added. Oligonucleotide sequences and primer-specific annealing temperatures of the primers are listed in Table 1. Primers were obtained from literature and subsequently confirmed in the National Center for Biotechnology Information Primer-BLAST database. Primer efficiencies were between 90% and 110%. Gene expression was determined using the ΔΔCq method of relative quantification where gene expression in the cells was normalized to GAPDH (human) or acidic ribosomal phosphoprotein P0 (Arbp) (rat). None of the hormonal or test compound treatments affected gene expression of the reference genes and coefficients of gene expression variation was below 5%.

Table 1. Primer sequences and primer-specific annealing temperatures used in this study.

	Encoding gene		5'	3'	Annealing Temperature (°C)
Human	GAPDH	FP	GAAGGTGAAGGTCGGAGTCAAC		60
		RP	CAGAGTTAAAAGCAGCCCTGGT		
	CYP1A1	FP	CAGAAGATGGTCAAGGAGCA		60
		RP	GACATTGGCGTTCTCATCC		
	AHR	FP	ACATCACCTACGCCAGTCGC		60
		RP	TCTATGCCGCTTGGAAGGAT		
	ERα	FP	CCACCAACCAGTGCACCATT		60
		RP	GGTCTTTTCGTATCCCACCTTTC		
	PR	FP	CGCGCTCTACCCTGCACTC		60
		RP	TGAATCCGGCCTCAGGTAGTT		
	IL-6	FP	GGTACATCCTCGACGGCATCT		60
		RP	GTGCCTCTTTGCTGCTTTCAC		
	IL-8	FP	TATGCACTGACATCTAAGTTCTTTAGCA		60
		RP	CTCTTGGCAGCCTTCCTGATT		
	MMP-9	FP	GGCTCCTGGCACACGCCTTT		57
		RP	TGGAACCACGACGCCCTTGC		
		RP	GACATGCCTGCGCTCTCATACTTA		
Rat	Arbp	FP	CCTAGAGGGTGTCCGCAATGTG		63
		RP	CAGTGGGAAGGTGTAGTCAGTCTC		
	Cyp1a1	FP	ATGTCCAGCTCTCAGATGATAAGGTC		60
		RP	ATCCCTGCCAATCACTGTGTCTAAC		
	Ahr	FP	TGGCTGTGATGCCAAAGGGCAG		59
		RP	AGCATGTCAGCGGCGTGGAT		
	ERα	FP	GGCTGCGCAAGTGTTACGAA		60
		RP	CATTTCGGCCTTCCAAGTCAT		
	PR	FP	TGGTTCCGCCACTCATCA		60
		RP	TGGTCAGCAAAGAGCTGGAAG		

2.5. Aromatase activity

CYP19 enzyme (aromatase) activity was measured in the endometrial cells after exposure to the compounds using the tritiated water-release method, as described previously [33]. CYP19 activity was measured as the amount of tritiated water formed after the conversion of the CYP19 substrate, radiolabeled [1β-3H]-androstenedione to estrone. Endometrial stromal and epithelial cells were exposed to laquinimod (0.1 μM-10 μM) DELAQ (0.1 nM-10 nM) and known inducers of CYP19 enzyme activity (100 nM phorbol 12-mystrate 13-acetate, 100 nM prostaglandin E2, 100 nM dexamethasone) or letrozole (3 μM) as control for inhibition of CYP19 enzymatic activity. After 2 days, medium was discarded and cells were incubated with 54 nM [1β-3H]-androstenedione for 6 h. After chloroform extraction and dextran-charcoal treatment, radioactivity was determined in the samples in disintegration per minute (dpm).

2.6. AHR CALUX assay

The human hepatoma HG2L7.5C1 CALUX cell line was created by a stable transfection of an AHR responsive firefly luciferase reporter gene plasmid pGudLuc7.5 under the control of dioxin-response elements [34], and was a kind gift of Prof. M. Denison (University of California, Davis, CA). The HG2L7.5C1 cells were cultured in MEMα medium (Invitrogen), supplemented with 10% FBS and 1% p/s. The cells were maintained at 37°C in 5% CO_2 and sub-cultured twice a week. The cells were seeded in 96-wells luciferase plates at a density of 40.000 cells/well. Cells were exposed to ranges of the AHR ligands or 0.1% v/v DMSO as vehicle control. After 24 h, the cells were lysed and mixed with luciferin reagent (pH 7.8) and luminescence was measured as a proxy for AHR activation. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays were performed to identify cytotoxicity. The AHR CALUX assay was performed in three independent experiments in triplicate.

2.7. Data analysis

Human gene expression data were collected from patient-paired co-cultures that were established from five independent endometrial isolations (donors). Rat experiments were performed four times. Within each experiment, exposures were performed in triplicate. The normalized gene expression data was calculated relatively to the vehicle control treatments within each experiment. The differences of the relative means for each treatment were analyzed using a Student's t-test. Differences of the means were considered to be statistically significant at $P \leq 0.05$.

3. Results

3.1. Physiological relevance of human endometrial model

Co-cultures of endometrial epithelial and stromal cells were exposed to different hormonal conditions to mimic the phases of the menstrual cycle. The physiological relevance of the human endometrial model was assessed at gene expression level of several marker genes. Chemokines play an important

role in the endometrium to facilitate embryo implantation. IL-6 and IL-8 are chemokines that are higher expressed in the secretory phase of the menstrual cycle, compared to the proliferative phase [35, 36]. Indeed, in our human endometrial model gene expression of IL-6 was on average 1.5-fold higher in the P4-dominant phase, compared to the E2-dominant phase (Figure 2). Gene expression of IL-8 was on average 1.9-fold higher in the P4-dominant phase, compared to the E2-dominant phase.

MMPs are involved in the degradation of the ECM surrounding the endometrial cells. Therefore, the expression of MMPs is highest at the onset of menstruation and low during the secretory phase of the menstrual cycle when P4 levels are highest [23]. Indeed, gene expression of MMP9 was 1.4-fold lower in the P4-dominant phase compared to the E2-dominant phase. Compared to the no hormone control phase, MMP9 expression decreased with an average of 1.4-fold in the E2-dominant phase, and an average of 2-fold in the P4 dominant phase.

Ki-67 is considered a proliferation marker and its expression increases when endometrial cells are in the proliferative phase of the menstrual cycle [37]. As endometrial cells are stimulated to grow under the influence of E2, it is expected that gene expression of Ki-67 will be highest in the endometrial cells that are in the E2-dominant phase. In our endometrial model, gene expression of Ki-67 increased on average 1.4-fold in the E2 dominant phase and on average 1.7-fold in the P4 dominant phase compared to the no hormone control.

Aromatase activity was assessed in three independent endometrial stromal cultures by the tritiated-water release assay. None of the tested cultures displayed aromatase activity (data not shown), even after treatment with known aromatase activity inducers prostaglandin E2 (100 nM), phorbol 12-myristate 13-acetate (100 nM) and dexamethasone (100 nM). Similar to the human endometrial stromal cells, no aromatase activity could be detected in rat endometrial stromal cells. Incubation with known inducers for aromatase activity did also not lead to detectable aromatase activity in the rat endometrial cells (data not shown).

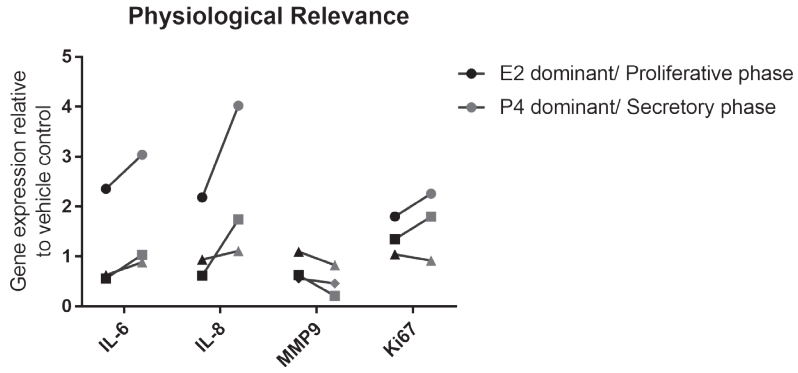

Figure 2. Gene expression of IL-6, IL-8, MMP9 and Ki67 in human co-cultures. Gene expression changes in individual human endometrial co-cultures in E2-dominant phase (black) and P4-dominant phase (gray) compared with no hormone treated cultures (set to 1). Gene expression was normalized using GAPDH as a reference gene. Individual co-cultures (N=3) are indicated with different symbols for every donor in each hormonal phase for all genes.

3.2. Human vs rat endometrial responses to hormones

In the human endometrial model, gene expression of ERα increased 2.0-fold when the endometrial model was exposed to the E2-dominant phase, and increased 1.5-fold when exposed to the P4-dominant phase although the expression was not statistically significantly different from vehicle-treated control cells (Figure 3A). While ERα expression tends to increase in human endometrial cells upon the exposure to hormones, the gene expression decreased statistically significantly by 2-fold in the rat endometrial cells in the P4 dominant phase (Figure 3B).

The change in PR gene expression in response to the hormonal phases was similar in both the human and rat endometrial models. In the human model, gene expression of PR statistically significantly increased 2.1-fold in the E2-dominant phase, and increased 1.7-fold in the P4-dominant phase (Figure 3A). In the rat endometrial model, PR gene expression statistically significantly increased 2.4-fold in the P4-dominant phase compared to the control (Figure 3B).

AHR gene expression changes differed between human and rat endometrial models following exposure to the different hormonal phases. Gene expression of the AHR did not change when the human endometrial model was exposed to the different hormonal phases compared to the vehicle-treated control (Figure 3A). In contrast, AHR gene expression statistically significantly decreased 2-fold when the rat model was exposed to the P4-dominant phase compared to the vehicle-treated control cells (Figure 3B).

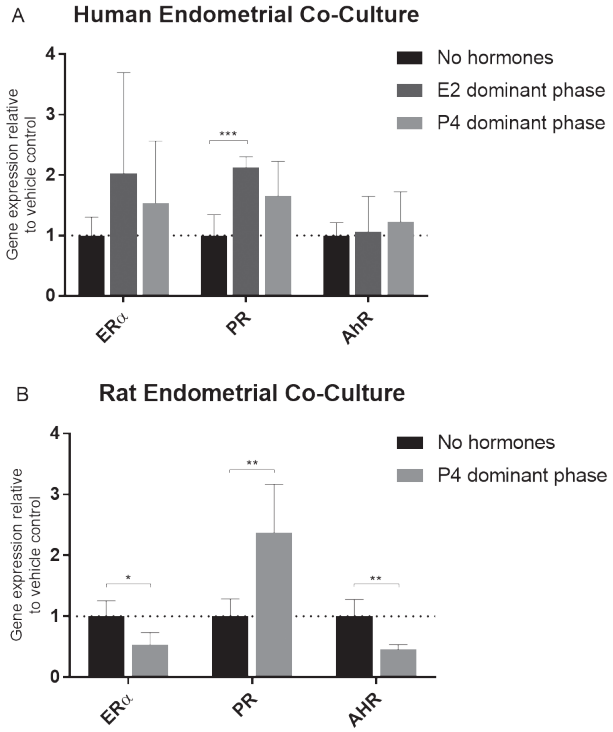

Figure 3. Nuclear receptor gene expression in human and rat endometrial co-cultures. Average gene expression of ER**α**, PR and AHR in human (A) and rat (B) endometrial co-cultures after exposure to an E2 dominant phase (human) and P4 dominant phase (human and rat). Gene expression was normalized with GAPDH (human) or Arbp (rat) as reference gene and expressed relative to the no hormone phase (set to 1). Bars represent 5 (human) or 4 (rat) individual donors/ independent experiments ± SD. Statistically significantly different from vehicle treated control with *P≤0.05, **P≤0.01 and ***P≤0.001 .

3.3. Effects of TCDD, laquinimod and DELAQ on human and rat endometrial cells

To gain insight into the relative potencies, the ability of TCDD, laquinimod and DELAQ to activate the AHR was assessed in an AHR reporter gene assay, using luciferase signal as a proxy for AHR activation. TCDD was the most potent AHR ligand in the human AHR luciferase reporter gene assay, followed by DELAQ and laquinimod (Figure 4). TCDD concentration-dependently induced the luciferase signal, with a statistically significant increase from 10 pM TCDD up. The concentration DELAQ needed to induce a similar response as 10 pM TCDD was approximately 100-fold higher, suggesting a relative potency of 0.01. Moreover, the efficacy of DELAQ to induce a luciferase signal was 2.9-fold lower than that of TCDD. Laquinimod only statistically significantly induced the luciferase signal at the concentration of 10 µM, which makes it ~10^6 less potent than TCDD in AHR activation.

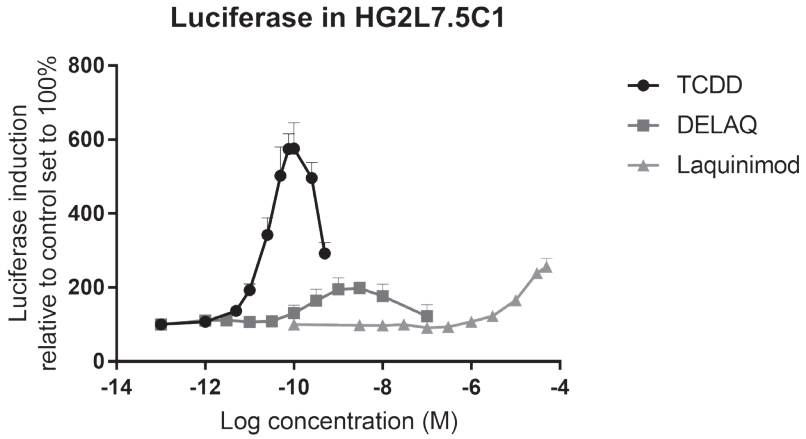

Figure 4. TCDD, DELAQ and laquinimod activate the AHR in human HG2L7.5C1 cells. TCDD (1 pM-0.5 nM), DELAQ (1 pM-100 nM) and laquinimod (3 nM-0.1 mM) induce luciferase after 24 h of exposure to the human AHR CALUX HG2L7.5C1 cells. Data is expressed as mean ± SD.

Next, the effects of the AHR ligands on the primary human and rat endometrial models under the different hormonal conditions were assessed using CYP1A1 gene expression as a proxy for AHR activation. Upon exposure to hormones, the AHR was differentially expressed in the human and rat endometrial cells (Figure 3). However, AHR gene expression did not change after exposure to TCDD, laquinimod, or DELAQ in the human and rat endometrial models (data not shown) in any of the hormonal conditions.

No statistically significant difference was observed between the hormonal conditions on TCDD-mediated induction of CYP1A1 gene expression in the human endometrial model (Table 2). Here, maximum induction of CYP1A1 gene expression by 1 nM TCDD was approximately 9.8-fold compared with vehicle-treated control cells (Table 3).

In the rat endometrial model however, the induction of CYP1A1 gene expression by 1 nM TCDD ranged between approximately 950-fold and 1500-fold with an average of 1191-fold compared to the vehicle-treated control cells under hormone-free conditions (Table 3). However when rat endometrial cells were exposed to TCDD under P4-dominant conditions, CYP1A1 gene expression was markedly attenuated and ranged between approximately 360-fold and 580-fold with an average of 429-fold compared to the vehicle-treated control cells. This statistically significant attenuation in CYP1A1 gene expression in the P4-dominant phase was on average 36 percent of that induced by TCDD with no hormones, *i.e*. an average of a 2.8-fold reduction (Table 2).

Laquinimod did not affect CYP1A1 gene expression in human or rat endometrial models up to 1 μM at any of the hormonal conditions in human (Figure 5A) or up to 10 μM in rat (data not shown). In contrast, DELAQ statistically significantly induced CYP1A1 gene expression in a concentration-dependent manner in both human and rat endometrial models at concentrations higher than 10 nM (Figure 5A).

DELAQ concentration-dependently induced CYP1A1 gene expression, which was already statistically significant at 0.01 nM in human endometrial cells under all hormonal conditions (Figure 5A). The average induction of CYP1A1 gene expression was similar for 0.1 nM DELAQ and 1 nM TCDD (Table 3), suggesting a roughly estimated relative potency of 10 for DELAQ in the induction of CYP1A1 gene expression in the human model. In the rat model, DELAQ also statistically significantly induced CYP1A1 gene expression in a concentration-dependent manner starting at 0.01 nM under hormone-free conditions (Figure 5B). However, this induction was ~3-times less potent compared to TCDD, suggesting a relative potency of 0.3 for DELAQ in induction of CYP1A1 (Table 3). Under P4-dominant conditions in rat endometrial cells, all DELAQ concentrations tested caused a statistically significant upregulation of CYP1A1 gene expression, but without clear concentration-dependency. The maximal induction of CYP1A1 gene expression by 1 nM TCDD was 122-fold lower in human than in the rat endometrial model. The maximal induction of CYP1A1 gene expression by 1 nM DELAQ was 51-fold lower in the human model compared to the rat model (Table 3).

Table 2. Human and rat TCDD-induced CYP1A1 gene expression relative to the no hormone situation. Average human and rat CYP1A1 gene expression induced by 1 nM TCDD relative to the no hormone situation per individual experiment N=5 (human) and N=4 (rat).

	HUMAN		RAT	
	AVR	*SD*	*AVR*	*SD*
No hormones	1	0.3	1	0.3
E2-dominant	1.01	0.4	NA	NA
P4-dominant	0.96	0.2	0.36	0.1

NA= Not Applicable

Table 3. CYP1A1 gene expression induction in human and rat endometrial co-cultures by TCDD and DELAQ. Data is expressed as average fold change (AVR) with standard deviation (SD) and biological replicates (N) relative to vehicle treated co-cultures.

		HUMAN			RAT					
		Hormonal conditions combined			No hormones			P4- dominant		
Treatment		*AVR*	*SD*	*N*	*AVR*	*SD*	*N*	*AVR*	*SD*	*N*
TCDD	0.1 nM	1.3	0.3	6	609	243	4	100	61	4
	1 nM	9.8	9.1	15	1191	212	4	429	103	4
DELAQ	0.01 nM	4.4	1.7	6	19	18	3	128	136	3
	0.1 nM	8.8	1.9	12	159	246	4	83	63	4
	1 nM	18.3	6.3	12	926	775	4	501	498	4
	10 nM	25.8	9.2	6	859	735	3	303	254	3

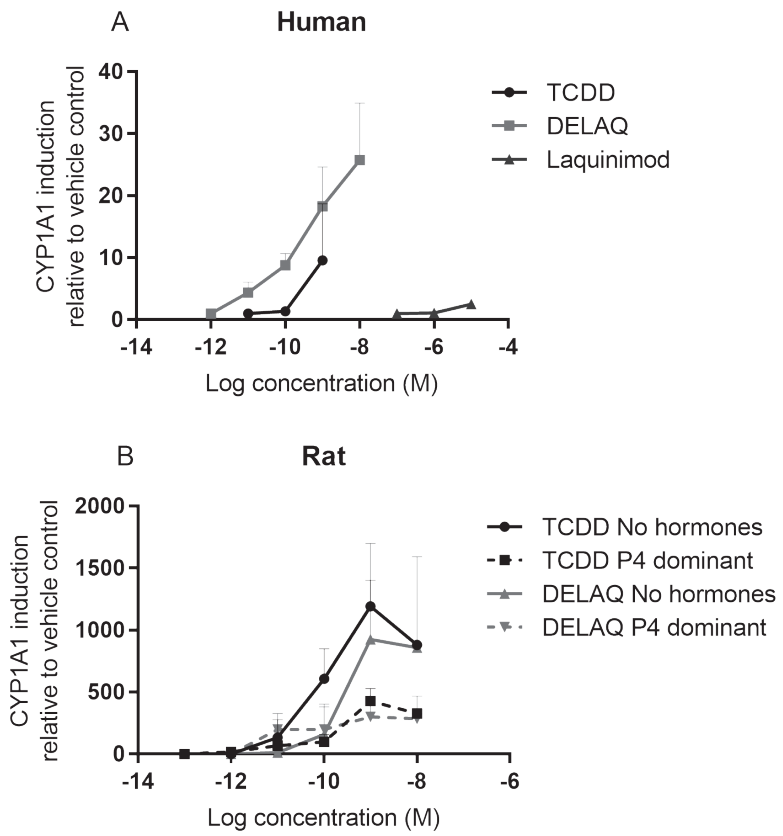

Figure 5. CYP1A1 gene expression in human and rat endometrial co-cultures by TCDD, laquinimod and DELAQ. A: Mean gene expression values and standard deviations for CYP1A1 gene expression relative to vehicle treated controls after exposure to 0.1-1 nM TCDD, 0.01-10 nM DELAQ, and 0.1-10 μM laquinimod in human endometrial co-cultures. Data for all the hormonal phases are pooled with a total N=15 as hormones did not affect CYP1A1 expression. Gene expression was normalized with GAPDH as reference gene. B: Mean gene expression values and standard deviations for CYP1A1 gene expression relative to vehicle treated controls after exposure to 1 pM-10 nM TCDD and 0.01-10 nM DELAQ in rat endometrial co-cultures. Data is expressed for every hormonal phase with total N=4. Gene expression was normalized with Arbp as reference gene.

4. Discussion

Clear differences were observed between primary human and rat endometrial 3D co-culture models in response to hormones alone and in combination with TCDD and DELAQ. AHR gene expression was not changed by hormones in the human model, while it was reduced in the rat model in the presence of hormones. This decrease in AHR gene expression coincided with an attenuated CYP1A1 induction by TCDD. Interestingly, DELAQ was a more potent AHR activator than TCDD in the human endometrial model, while DELAQ was less potent than TCDD in the rat endometrial model.

4.1. Physiological relevance of the endometrial model

A human 3D endometrial co-culture model was established by employing co-cultures of patient-paired primary epithelial and stromal endometrial cells with different hormonal conditions to mimic the proliferative (E2-dominant) and secretory (P4-dominant) phases of the human menstrual cycle. To assess the physiological relevance of the model, gene expression of several marker genes was determined. In the human model, higher gene expression of chemokines IL-6 and IL-8 was observed in the P4-dominant phase compared to the vehicle-treated control. This is in line with literature describing that gene expression of IL-6 and IL-8 is highest at the end of the secretory phase of the menstrual cycle [35, 36]. In addition, Marbaix and coworkers [23] reported that P4 blocks the secretion and activation of MMPs in human endometrial explants, which corresponds to the findings in our study and is in line with the concept that P4 controls *in vivo* endometrial breakdown. Gene expression of Ki-67 was also induced by hormones in the endometrial model, but did not differ between the experimental hormonal phases. Finally, it is generally acknowledged that aromatase expression and activity in endometrium is restricted to women with endometriosis, leiomyomas and adenomyosis [38]. The lack of aromatase activity in the human endometrial cells further supports the notion that our model is representative for the healthy human endometrium.

ER expression is a classical marker of E2-responsiveness in healthy endometrium. This expression is highest when circulating levels of E2 are high, *i.e.* during the proliferative phase of the menstrual cycle [39, 40]. Indeed, increased ERα gene expression was observed in the human endometrial model in the E2-dominant phase. This is also in line with results from Prange-Kiel *et al*. (2001) who showed that E2 increased ER receptor expression in human endometrial

cultures, while co-treatment with P4 reduced this expression [41]. The increased ERα expression in the P4-dominant phase in our study may therefore be explained by the relatively low concentration of E2 still present, and possibly a P4 concentration that was not high enough to inhibit the E2-induced ERα increase. In rats, it has also been reported that E2 induces ERα gene expression in the endometrium [42]. The rat model in our study was not exposed to an E2-dominant phase. In contrast to the human model, ER expression was reduced in the rat model in the P4-dominant phase. The results from our study may indicate that P4 exerts a stronger inhibitory effect in the rat endometrial cells compared to the human cells. Similar findings have been reported by Medlock *et al*. (1994) who found that progesterone down-regulates the ER in rat uterine tissue even when *in vivo* estrogen levels are high [43].

Endometrial PR expression in humans and rats is predominantly regulated by E2, and E2-induced PR expression can be attenuated by P4 in both species [42, 44]. In the human endometrial model, a higher PR induction was indeed observed in the E2-dominant phase. The PR induction in the P4-dominant phase in both models can be explained by the low concentration E2 present in that phase. It seems that the concentration P4 in our study counteracts the E2-induced PR expression. This suggests that in our model, PR expression is predominantly regulated by E2, while ERα expression is regulated by E2 but also *via* an inhibitory mechanism by P4 in rats, but not in humans.

A previous study described that AHR gene expression in human endometrial explants does not differ between the proliferative and secretory phases [45]. These findings are similar to our finding that AHR gene expression does not change in human endometrial cells when exposed to hormones. In contrast, reduced AHR gene expression levels were found in female Wistar rats that were treated with P4 *in vivo* [46], which is also in line with our *in vitro* findings.

Our results show a large human inter-individual variability in response to hormones, especially in the E2-dominant phase. It is possible that the phase of the menstrual cycle of the individual donors affected the observed quantitative responses, despite the wash out period that could be expected through *in vitro* culture of the cells. Unfortunately, no information was available regarding the donor's phase of the menstrual cycle at the time of surgery. However, the increase of both ERα and PR gene expression and the response to other physiological relevant marker genes in our endometrial model indicates an *in vivo*-like response for both human and rat endometrial cells. Combined with the lack of aromatase activity in the endometrial model, this indicates that the human and rat endometrial models may indeed be considered as relevant endometrial *in vitro* models.

4.2. Species differences in AHR response

In the human endometrial model, large interindividual variation was observed in the induction of CYP1A1 gene expression by TCDD. This was in line with what was expected, as individual variation in sensitivity to AHR activation can be caused by *e.g.* genetic differences and/or lifestyle, such as smoking [32, 47, 48].This variation may also be explained by different menstrual status of the donors. Nonetheless, similar trends with respect to the induction of CYP1A1 gene expression by TCDD and DELAQ were observed for every donor.

The induction of CYP1A1 gene expression by TCDD was similar to that observed by others using endometrial explants or primary human immortalized endometrial cells [49, 50]. However, in contrast to Willing and coworkers [49], we did not observe an attenuation of TCDD-mediated CYP1A1 gene induction by E2. This difference might be explained by our use of a co-culture with human epithelial and stromal cells *versus* immortalized human epithelial cells, or different hormonal exposure scenarios. In the rat endometrial model, an attenuated induction of CYP1A1 gene expression by TCDD was observed in the P4-dominant phase. Most likely, this was due to the observed decrease in AHR gene expression in the P4-dominant phase in these cells.

We observed that the relative potency of CYP1A1 induction by DELAQ was higher than TCDD in the human model, while it was lower than TCDD in rat. This may result from significant amino acid variations in the ligand binding domains of the AHRs from these species that contribute to significant differences in ligand binding specificity and species-differences in ligand response [51, 52]. Additionally, in contrast to the human endometrial model, TCDD was more effective than DELAQ in the human liver CALUX cell line. Differential ligand binding by both compounds could lead to significant differences in the overall structure of each ligand:AHR complex that allow differential recruitment of co-activators and interaction with nuclear factors present in each cell type, leading to the observed differences in potency and efficacy of the induction response responsiveness [13]. Furthermore, a lower fold induction of CYP1A1 in human endometrial cells was observed compared to those of the rat. This

observation is in agreement with other studies that have reported that rats are more sensitive to AHR activation of gene expression than humans, and this has been suggested to result from differential expression and recruitment of co-activators [53, 54]. The species-difference in AHR activation and CYP1A1 induction may also be explained by the higher basal CYP1A1 expression in human endometrial cells. However, absolute AHR and CYP1A1 basal gene expression data in human and rat endometrial cells was not determined in this study and the interpretation of results should therefore be considered with caution.

Persistent activation of the AHR has long been believed to account for the adverse effects of dioxins. A variety of AHR agonists have however demonstrated to induce AHR-dependent genes without causing the toxicity that dioxins are known for [55]. It has been reported that AHR activation can lead to significant anti-estrogenicity via multiple mechanisms, including CYP1A1-mediated breakdown of E2 and downregulation of ER signaling *via* AHR-ER cross-talk and interaction with selected ER responsive elements on the DNA [9, 13]. Our observation that DELAQ causes significant AHR activation in human endometrial cells may imply that this compound may exhibit anti-estrogenic properties in these cells, an effect that is considered to be associated with anti-carcinogenic properties.

5. Conclusion

The differences in regulation and activation of the AHR between human and rat endometrial cells can significantly impact the translation of toxicological findings from *in vivo* rat models to the human situation. This can have great consequences when performing risk assessment of chemicals, including pharmaceuticals, focused on female reproductive health. Our findings therefore underline the importance of using a human relevant *in vitro* model to determine potential effects of compounds on endometrial health.

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8. Declaration of interest

Declarations of interest: none.

9. References

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Chapter 3

Assessing anti-estrogenic effects of AHR ligands in primary human and rat endometrial epithelial cells

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Abstract

Unopposed estrogenic action in the uterus can promote the development of endometrial cancer in humans and rats. Aryl hydrocarbon receptor (AHR) activation gives rise to anti-estrogenic actions and may consequently reduce the development of endometrial cancer. In this study, the anti-estrogenic potential of the AHR ligands 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and DELAQ, a metabolite of the pharmaceutical laquinimod, was assessed in primary human and rat endometrial epithelial cells (EECs) with and without co-exposure to endogenous hormones.

In human EECs, estradiol and progesterone did not affect AHR gene expression, but in rat EECs, progesterone decreased AHR expression (1.4-fold). In accordance, AHR-mediated induction of CYP1A1/1B1 expression by DELAQ and TCDD decreased in hormone-treated rat EECs. DELAQ was 33-fold more potent than TCDD in human EECs in inducing CYP1A1/1B1 gene expression, while DELAQ was approximately 16-33-fold less potent than TCDD in rat EECs. In human EECs,10 nM DELAQ decreased estradiol-induced expression of growth-regulated estrogen receptor binding 1 (GREB1) by 1.8-fold,. In rat EECs, both DELAQ and TCDD did not affect the expression of estradiolinduced genes.

This study shows that AHR ligand DELAQ, but not TCDD, causes anti-estrogenic effects in primary human EECs. Furthermore, although AHR-mediated CYP1A1/1B1 induction by DELAQ and TCDD was stronger in rat EECs than human EECs, this did not result in apparent anti-estrogenic effects in the rat cells. This study shows that primary human and rat endometrial cells respond differently towards hormones and AHR ligands. This should be considered in human risk assessment based on rodent studies.

1. Introduction

Endometrial cancer is the most common gynecologic malignancy in women in developed countries that most often affects postmenopausal women [1]. A possible mechanism by which endometrial cancer may arise is via a disrupted estrogen (E2)/progesterone (P4) ratio in the uterus [2, 3]. These steroids are produced in the ovaria and the levels are tightly regulated throughout the menstrual and estrous cycle. E2 stimulates the epithelial endometrial cells to proliferate in order to prepare for implantation of a blastocyst. An aberrant hormonal (E2/P4) environment can therefore cause a variety of adverse effects in the uterus. Indeed, increased circulating E2 levels have been linked to a higher incidence of uterine carcinomas in animal experiments as well as in human epidemiological studies [4, 5]. While spontaneous endometrial cancer is rare in young naïve rodents, endometrial adenocarcinomas are a common phenomenon in aged rats and in rat toxicity studies with environmental contaminants and pharmaceuticals [2, 6]. As opposed to rat toxicity studies, epidemiological studies and clinical trials typically do not reveal a clear association between these compounds and endometrial tumors in humans. One mechanistic explanation for the endometrial cancer risk could be that estrogen signaling can be modulated through cross-talk with the aryl hydrocarbon receptor (AHR) [7, 8]. Also, activation of the AHR can display species-specific responses depending on the AHR ligands [9]. AHR activation by both endogenous and exogenous compounds can result in the induction of cytochrome P450 (CYP) enzymes. In the human endometrium, E2 is metabolized by cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1) and AHR activation can therefore result in local metabolism of E2. AHR activation has also been shown to result in other anti-estrogenic effects by interfering with estrogen receptor (ER)-DNA binding to specific estrogen responsive elements that contain an overlapping dioxin response element sequence. This can lead to stimulation of ER proteasomal degradation, inhibition of selected E2-target genes and other as yet unidentified mechanisms [10]. The AHR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been shown to exert anti-estrogenic effects by reducing the incidence of E2-dependent tumors in rat xenograft models *in vivo*, including uterine tumors, and the E2-induced proliferation of endometrial Ishikawa and breast tumor MCF-7 cell lines *in vitro* [11–13].

A previous study by our group showed that TCDD and DELAQ are potent inducers of CYP1A1 gene expression in primary cultures of human and rat endometrial cells, albeit with apparent species differences [14]. Considering this potency to activate the AHR, an inhibitory cross-talk with estrogen signaling is expected in human and rat endometrial cells. The present study was designed to assess the potential anti-estrogenic effects of TCDD and DELAQ in primary human and rat endometrial epithelial cells (EECs) and compare the effecs between both species.

2. Methods

2.1. Primary tissue and isolation

Endometrial tissue of eight individual human donors was obtained from the St Antonius hospital in Nieuwegein, the Netherlands. The study was approved by the hospital's Medical Ethical Committee (Study number Z15.038) and written informed consent for the use of endometrial tissue was obtained from all patients. None of the donors were using hormonal medication prior to or at the time of hysterectomy.

Rat endometrial cells were isolated from ~7-week old female Sprague-Dawley rats under approval of the Dutch law for animal welfare and protocols were approved by the Ethical Committee on Animal Research and the Animal Care Committee of Utrecht University (AVD108002015135; WP 800-15-135-01-002).

The human and rat endometrial epithelial cells were isolated as described earlier [14]. Both human and rat EECs were cultured in RMPI-1640 medium + 1% penicillin/streptomycin (p/s) + 10% fetal bovine serum (FBS; Hyclone, GE Healthcare) and kept in an incubator at 37 °C in 5% CO2. Medium was refreshed every 3 days.

2.2. Experimental conditions

It has been documented that human EECs cultured on a layer of extracellular matrix (ECM) grow in a polarized, more *in vivo* like fashion [15]. Therefore, both human and rat EECs were seeded on pre-coated ECM 24-well plates. The coating comprised of a thin layer of 1:1 medium and ECM (Sigma, the Netherlands) that was allowed to polymerize at 37 °C. After ~7 days of culture, the epithelial cells were detached from the culture flasks using Accutase (Life Technologies) and seeded with a density of approximately 90.000 cells per well. The culture medium was replaced with RMPI-1640 medium + 1% p/s + 10% dextran charcoal treated FBS (Hyclone, GE Healthcare). Cells were seeded with or without hormones: 0.1 % ethanol (no hormones), 100 nM estradiol (E2; Sigma) or 100 nM progesterone (P4; Sigma). After 3 days, medium was replaced with medium containing hormones and different concentrations of TCDD, DELAQ, or 0.1% DMSO as a vehicle control. After 48h of exposure, RNA Instapure (Eurogentec, Belgium) was added to the cells to preserve the mRNA. Cells were stored at -80 °C upon RNA isolation.

2.3. RNA isolation and qPCR

RNA was isolated by phenol–chloroform extraction method. 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, The Netherlands), 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 using the CFX96 cycler (BioRad). A PCR mastermix was made containing IQ SYBR Green Supermix, forward and reverse primer with a concentration of 10 µM and RNA-se free water. Samples were heated initially at 95 °C for 15 minutes and then followed by 40 cycles with denaturation at 94 °C for 30 seconds, annealing/extension temperature (between 55 and 65°C) for 30 seconds. Oligonucleotide sequences and primer-specific annealing temperatures of the human and rat are listed in Table S1. Primer efficiencies were established as between 90% and 110%. Gene expression was determined using the ΔΔCq method of relative quantification where gene expression was normalized to GAPDH and β-actin (human samples) or Arbp and β-actin (rat samples).

2.4. Data analysis

Experiments were performed in triplicate, unless stated otherwise. Unpaired t-tests were used to analyze the statistical difference of the mean between the hormone treatments in the vehicle controls of human and rat EECs. Concentration-response curves for CYP1A1 and CYP1B1 induction were calculated relative to 1 nM and 0.1 nM TCDD for human and rat, respectively. Symbol meaning; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

3. Results

3.1. Hormone responses in human and rat EECs

In humans and rats, hormonal regulation of cyclic endometrial growth is regulated through ER and progesterone receptor (PR). In a preliminary experiment, ERα gene expression was not consistently induced by E2 in the human EEC, whereas ERβ gene expression was very low in human EECs, and did not respond to E2 treatment (data not shown). Therefore, the gene expression of both isoforms of the ER was not included in the assessment of anti-estrogenic effects in this study. In contrast, gene expression of PR and growth-regulated estrogen receptor binding 1 (GREB1) showed clear E2-responsiveness in human EECs. PR gene expression was statistically significantly 1.3-fold higher in the E2-treated human EECs, compared to the vehicle-treated control cells (Figure 1A). GREB1 gene expression was statistically significantly 7.8-fold higher in the E2-treated human ECCs, compared to the vehicle-treated control cells (Figure 1B). The expression of E2-responsive genes PR and GREB1 was not affected by P4 in human EECs. AHR gene expression did not change in the E2-treated or P4-treated human EECs, compared to the vehicle-treated control cells (Figure 1C).

Similar to human EECs, ERα gene expression was not consistently and strongly induced by E2 treatment in rat EECs in this study (data not shown). PR gene expression also showed clear E2-responsivess in rat EECs and was statistically significantly 2.7-fold higher in the 100 nM E2-treated cells, compared to the vehicle-treated control cells (Figure 2A). As gene expression of GREB1 in rat uterine tissue has not been reported in literature, heat shock protein 27 (HSP27) gene expression was also investigated as E2-responsive gene in rat. In rat EECs, HSP27 gene expression was statistically significantly 1.6-fold higher in the E2-treated cells compared to the vehicle control treated cells (Figure 2B). mRNA expression of E2-responsive genes, PR and HSP27, was not affected by P4 in rat EECs. In contrast to human EECs, AHR gene expression was affected by hormones in rat EECs. AHR gene expression slightly decreased 1.1-fold upon treatment with E2, and statistically significantly decreased 1.4-fold upon treatment with P4 (Figure 2C).

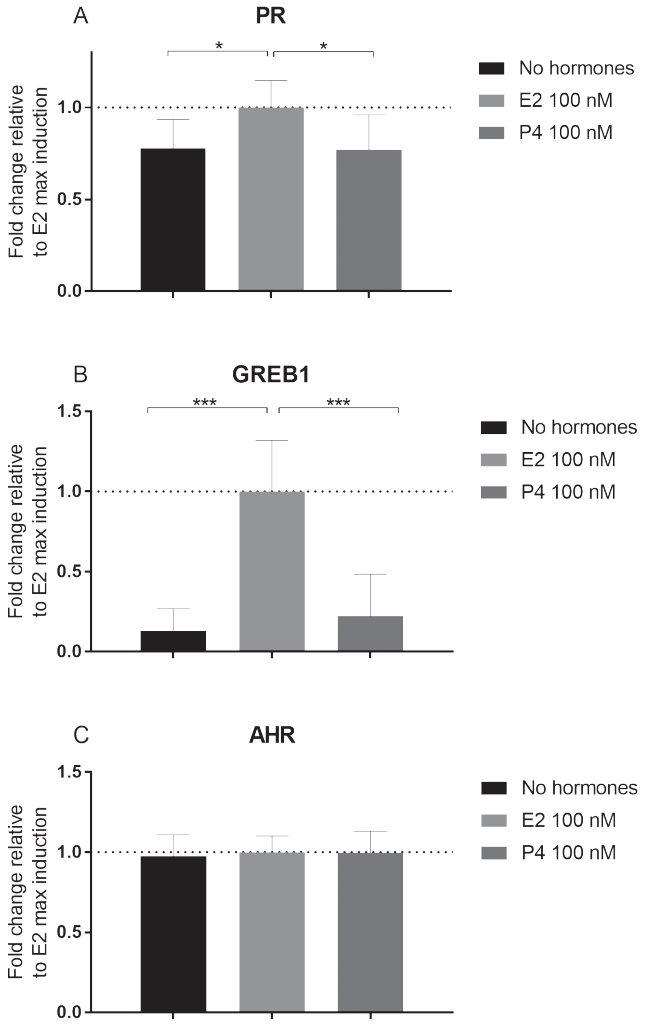

Figure 1. Effect of hormones (no hormones (0.1% v/v ethanol), 100 nM estradiol (E2) or 100 nM progesterone (P4)) on the expression of A) PR, B) GREB1 and C) AHR mRNA in human endometrial epithelical cells (EECs). Values are expressed as mean values and standard deviations relative to the expression in E2-treated human EECs with N=8. Statistically significantly different with * P≤0.05 and *** P≤0.001.

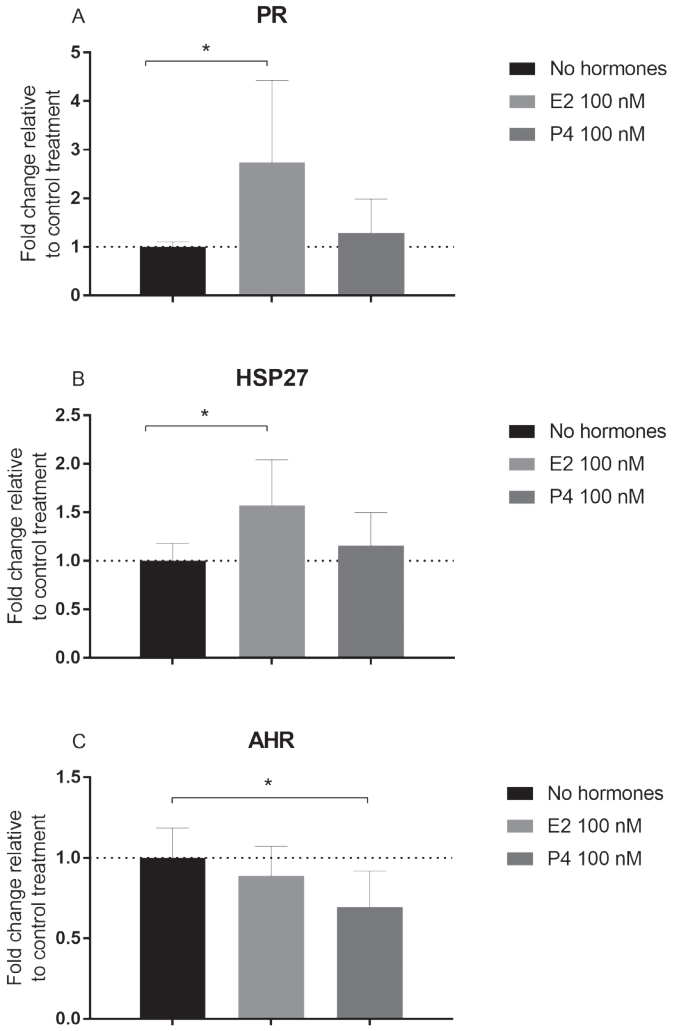

Figure 2. Effect of hormone treatment (no hormones, 100 nM estradiol (E2) or 100 nM progesterone (P4)) on the expression of A) PR, B) HSP27 and C) AHR mRNA in rat EECs. Values are expressed as mean values and standard deviations relative to the expression in vehicle treated (0.1% ethanol) control cells with N=5. Statistically significantly different from no-hormone treated EECs with * P≤ 0.05; *** P≤ 0.001.

3.2. AHR gene battery in human and rat EECs

To determine the effects of TCDD and DELAQ on AHR-dependent genes, CYP1A1 and CYP1B1 were studied in human and rat EECs. In human EECs, AHR gene expression did not statistically significantly change upon 1 nM TCDD or 0.01-10 nM DELAQ treatment alone or in combination with E2 or P4 (data not shown).

Normalized CYP1A1 gene expression induced by TCDD varied from 1.2-fold to 17.8-fold between donors (data not shown). To correct for interindividual differences in CYP1A1 inducibility, changes in CYP1A1 gene expression are displayed relative to CYP1A1 gene expression as induced by 1 nM TCDD for every individual experiment. CYP1A1 gene expression induced by TCDD was not affected by E2 or P4 in human EECs (data not shown). CYP1A1 gene expression was statistically significantly induced by DELAQ in a concentration-dependent manner compared to the vehicle control, and was not affected by either E2 or P4. In the human EECs, the induction of CYP1A1 gene expression by 1 nM TCDD was equipotent to 0.03 nM DELAQ (Figure 3A, intersection of dotted line and fitted curve). Therefore, the mean relative effect potency of DELAQ to TCDD for CYP1A1 expression in human EECs is ~33.

CYP1B1 gene expression induced by 1 nM TCDD was less variable between donors than CYP1A1 gene expression, with an interindividual ~4-fold variability (data not shown). The maximal induction of CYP1B1 gene expression by 1 nM TCDD in human EECs was 1.5-fold lower than the induction of CYP1A1 for all the hormonal conditions combined (data not shown). Similarly to CYP1A1, CYP1B1 gene expression was not affected by E2 or P4. However, the gene expression of the endometrial cells treated with 10 nM DELAQ in combination with P4 deviated from the other treatment groups. With this hormonal treatment, gene expression was 1.8-fold higher compared to the no hormone treatment. CYP1B1 gene expression was statistically significantly induced by 0.01-10 nM DELAQ in a concentration-dependent manner. Similar to CYP1A1 induction, the mean relative effect potency of DELAQ to TCDD for CYP1B1 gene expression in human EECs was ~33.

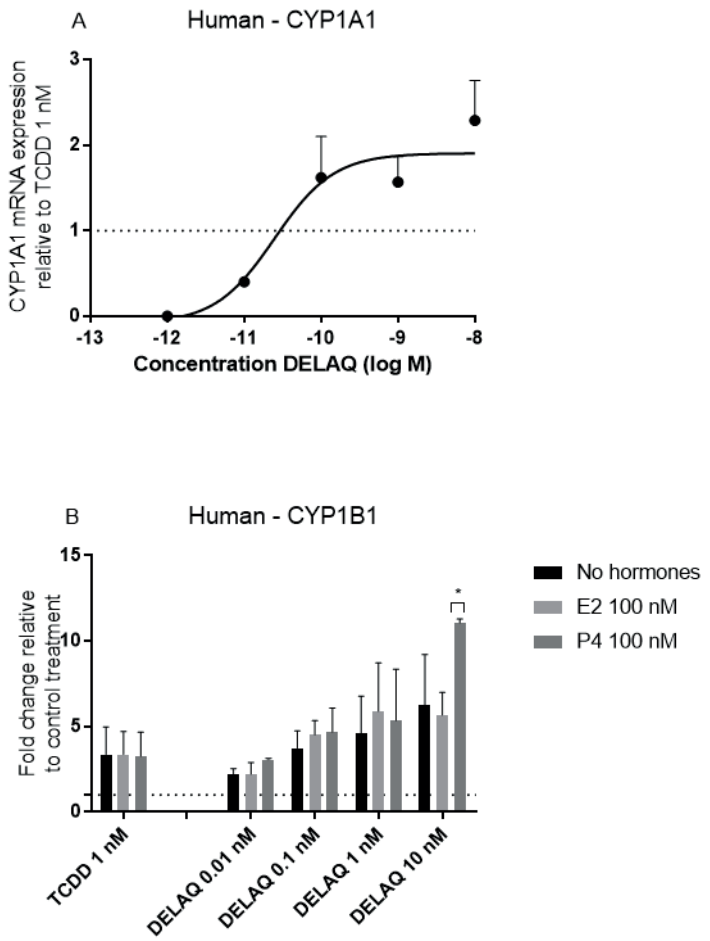

Figure 3. mRNA expression of CYP1A1 (A) and CYP1B1 (B) in human EECs. Data is expressed relative to 1 nM TCDD (CYP1A1) or relative to the vehicle (0.1% DMSO) treated control cells (CYP1B1) with total N=7.

In rat EECs, basal CYP1A1 gene expression was extremely low in the vehicle-treated control EECs. As a result, normalized CYP1A1 gene expression induced by 0.1 nM TCDD varied from 10-fold to 740-fold in the P4 exposed cells, when calculated relative to the vehicle-treated control EECs. Therefore, changes in CYP1A1 gene expression were calculated relative to CYP1A1 gene expression induced by 0.1 nM TCDD.

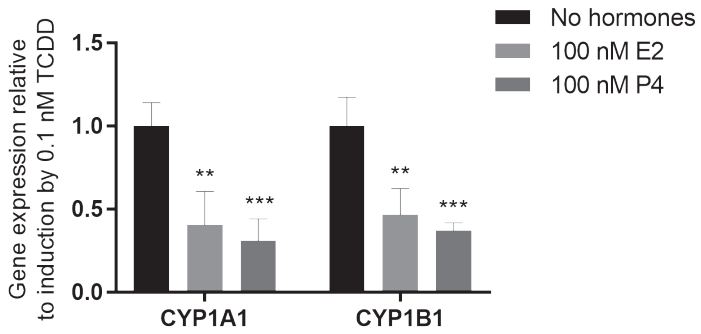

Figure 4. mRNA expression of CYP1A1 and CYP1B1 in rat endometrial epithelial cells. Data is expressed relative to induction by 0.1 nM TCDD in the no hormone (0.1% ethanol) treatment with N=4. Statistically significantly different from no-hormone treated EECs with ** P≤ 0.01; *** P≤ 0.001.

CYP1A1 gene expression induced by 0.1 nM TCDD statistically significantly decreased 2.5-fold after co-exposure with E2 and 3.1-fold after co-exposure with P4 (Figure 4). CYP1A1 gene expression was induced by both TCDD and DELAQ in a concentration-dependent manner in rat EECs (Figure 5). In rat EECs, the induction of CYP1A1 gene expression by 0.1 nM TCDD was equipotent to 2.1 nM DELAQ in the vehicle-treated control cells, and 1.6 nM in the E2 and P4 treated cells (Figure 5A intersection dotted line and fitted curves). The mean relative effect potency of DELAQ to TCDD for induction of CYP1A1 expression in rat EECs compared to TCDD is ~0.05 in the no hormone treatment and ~0.06 in the E2 and P4 treatments.

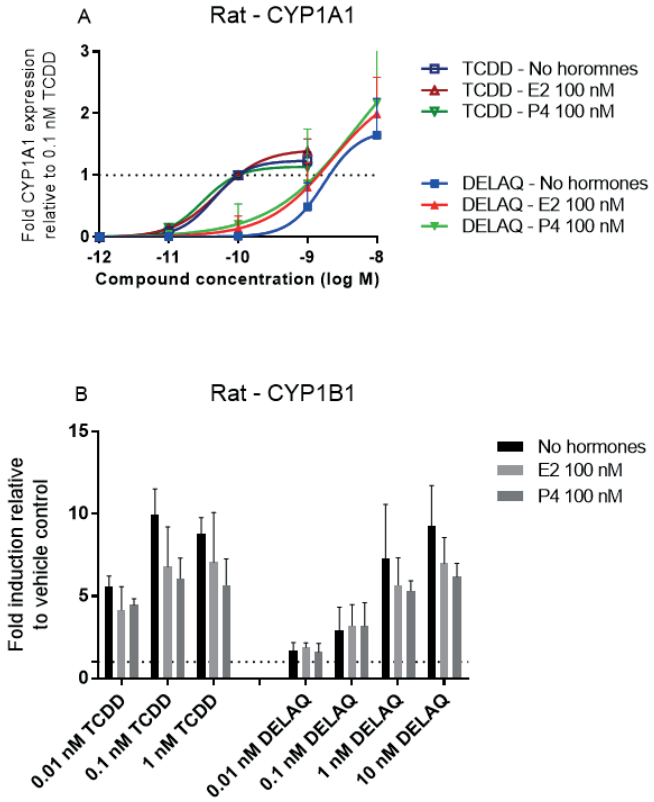

Figure 5. mRNA expression of CYP1A1 (A) and CYP1B1 (B) in rat endometrial epithelial cells. Data is expressed relative to 0.1 nM TCDD treated cells for every hormonal treatment with total N=4.

The maximal induction of CYP1B1 gene expression by 0.1 nM TCDD in rat EECs was approximately 26-fold lower than the induction of CYP1A1 in the no hormonal phase. CYP1B1 was induced in a concentration dependent manner from 0.01-1 nM TCDD and 0.1-10 nM DELAQ. Similarly to CYP1A1, CYP1B1 gene expression induced by 0.1 nM TCDD statistically significantly decreased 2.2-fold after co-exposure with E2 and 2.7-fold after co-exposure with P4 (Figure 5). The induction of CYP1B1 gene expression by 0.1 nM TCDD was equipotent to 1.8 nM DELAQ in the E2 treatment and 3.5 nM in the P4 treatment as calculated by expressing CYP1B1 gene expression relative to the treatment of 0.1 nM TCDD (data not shown). No comparison could be made for the no hormone treatment, as CYP1B1 induction by DELAQ up to 10 nM did not reach the level of 0.1 nM TCDD-induced CYP1B1 gene expression in the no hormone treatment. The relative effect potency of DELAQ to TCDD for CYP1B1 gene expression in the rat EECs is ~0.06 in the E2 treatment and ~0.03 in the P4 treatment.

3.3. Human and rat EECs and anti-estrogenicity

Activation of the AHR can result in an inhibitory cross-talk with estrogen signaling pathways. To determine anti-estrogenic potential, the effects of AHR ligands TCDD and DELAQ on E2-responsive genes was assessed in human and rat EECs.

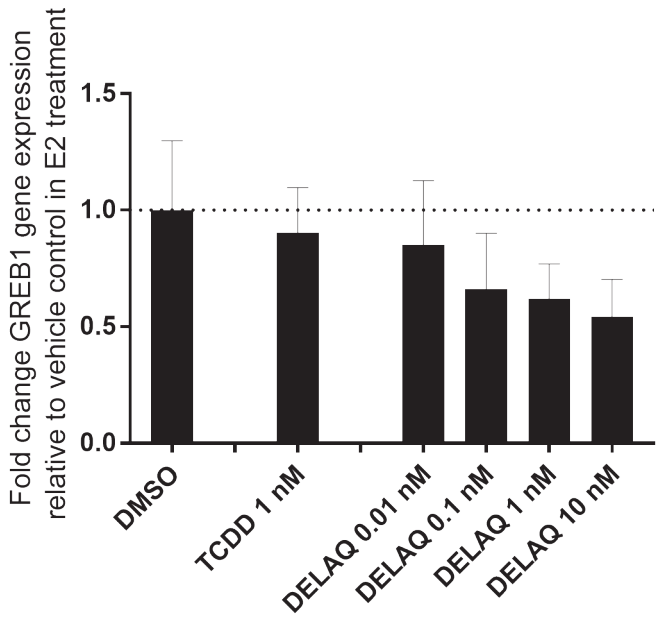

Figure 6. mRNA expression of GREB1 in human endometrial epithelial cells. Data is expressed relative to the induction in the 100 nM E2 treaded vehicle-treaded control cells. N=6 with N=3 for TCDD, N=2 for 0.01 nM and 10 nM DELAQ and N=3 for 0.1 and 1 nM DELAQ.

Although a slightly decreasing trend in PR gene expression was observed after exposure to DELAQ in E2-treated human EECs, both TCDD or DELAQ did not cause a statistically significant reduction of E2-induced PR gene expression nor in the other hormonal treatments (data not shown). In our study, E2 alone increased PR gene expression 1.3-fold, but increased GREB1 gene expression 7.8-fold (Figure 1). Here, anti-estrogenic effects on GREB1 expression were observed in human EECs following treatment with DELAQ (Figure 6). DELAQ reduced E2-induced gene expression of GREB1 in a concentration-dependent manner. E2-induced GREB1 expression decreased by 1.6-fold and 1.8-fold by 1 and 10 nM DELAQ treatment, respectively. Yet, this effect was not considered statistically significant as P-values were 0.06 for both 1 and 10 nM DELAQ. TCDD (1 nM) did not statistically significantly affect the gene expression of GREB1 induction by E2 (Figure 6). In contrast to the human EECs, no changes in the gene expression of E2-responsive genes PR and HSP27 were observed in rat EECs after treatment with TCDD or DELAQ (data not shown).

4. Discussion

This study showed clear species-differences in AHR-mediated responses in human and rat primary EECs. Firstly, E2 and P4 reduced AHR and CYP1A1 gene expression in rat, but not in human EECs. Secondly, maximal induction of AHR-responsive genes was higher in rat EECs compared to human EECs. Secondly, anti-estrogenic effects of DELAQ were only apparent in human EECs, and not in rat EECs. Furthermore, exposure to the best-known AHR agonist TCDD did not show any anti-estrogenic effect in primary human or rat EECs. The relative effect potency for AHR activation by DELAQ was higher than TCDD in human EECs. This may explain why anti-estrogenic effects of AHR activation were only apparent in human EECs upon DELAQ exposure, and not in rat EECs. These fundamental species-differences should be considered when assessing endometrial tumor development in rat studies and its relevance for the human situation.

Endometrial tumors are considered a consequence of disbalance between estrogen and progesterone in the uterus. Estrogen metabolism plays a crucial role herein as it affects estrogen levels. AHR activation can modulate estrogen metabolism *e.g.* via the induction of estrogen metabolizing enzymes like CYP1A1 and 1B1. The AHR is constitutively expressed in the human endometrium. In this study, AHR gene expression did not change in primary human EECs in vitro upon E2 or P4 treatment. This is in line with several studies showing no changes in AHR gene expression in endometrial tissue obtained during follicular and luteal phases of the female menstrual cycle [16, 17]. In contrast, in rat EECs, AHR gene expression decreased upon treatment with E2 and P4. This decrease was also observed in a previous study performed by us using a co-culture of primary epithelial and stromal cells after exposure to hormones [14]. Rataj et al. also described that AHR mRNA was downregulated by both E2 and P4 in uteri of ovariectomized rats *in vivo* [18, 19]. The authors described a stronger effect of E2 on AHR downregulation than P4, while in the present study, P4 exerted a stronger effect on AHR downregulation in rat EECs *in vitro*. Possibly, the uteri of ovariectomized rats respond stronger to E2 which can account for the differences between the studies. Chaffin et al. reported a decrease in AHR gene expression in ovarian cells in Lewis rats *in vivo* during the pro-estrus, when both E2 and P4 levels are high [20]. These data combined indicate a species-specific difference in AHR expression rather than a cell type specific effect in response to hormones.

Besides the differences in AHR expression, also species-specific differences in AHR activation was observed in our study. Here, we demonstrate that DELAQ is more potent than TCDD in CYP1A1/1B1 activation in human endometrial cells, while the reversed was observed in rat cells. The relative potencies of DELAQ compared to TCDD to induce CYP gene expression was on average, approximately 0.05 in rat and 33 in human EECs. These species differences in AHR ligand response may result from differences in ligand binding domains of the AHR which can result in ligand binding specificity [21, 22].

CYP1A1 and CYP1B1 are considered AHR-responsive genes, but the CYP enzymes also play an important role in estrogen metabolism thus normal physiology. Similarly to present study, a large interindividual variability in CYP1A1 and CYP1B1 gene expression patterns was reported in various human primary endometrial cell types [17]. In addition, Bulun et al. did not find any difference in basal CYP1A1 and CYP1B1 gene expression between uterine samples from the proliferative and luteal phases. This is in line with our observation that in human EECs, CYP1A1 and CYP1B1 gene expression was not affected by E2 or P4 exposure. Remarkably, studies have demonstrated an estrogen responsive element (ERE) in the CYP1B1 promotor, suggesting a stimulatory effect of E2 on CYP1B1 [23]. Possibly, the effect of E2/ERE on CYP1B1 induction is tissue-

specific, as this was not observed in our study [24]. However, to the best of our knowledge, no studies have investigated endometrium-specific regulation of CYP1B1 or the role of CYP1B1 in ER regulation in primary endometrium.

Despite species-, ligand-, and tissue differences in AHR-mediated effects, typically, CYP1A1 is more highly induced than CYP1B1 upon AHR activation [25–27]. The preferential induction of CYP1A1 over CYP1B1 was also observed in human and rat EECs in this study. The differential induction of CYP1A1 and CYP1B1 is especially important, when considering the role of these CYPs in estradiol metabolism [28]. CYP1A1-mediated metabolism leads to predominantly 2-hydroxyestrogen formation, which is considered the "good" metabolite. CYP1B1-mediated estrogen metabolism, in contrast, results in formation of the potentially carcinogenic metabolite 4-hydroxyestrogens [7, 29, 30]. Based on normalized gene expression, the CYP1A1/1B1 ratio upon AHR activation by TCDD or DELAQ was approximately 1 in human EECs, but this was approximately 0.5 and 0.7 in rat EECs for TCDD and DELAQ respectively. This suggests a shift in estrogen metabolism, favoring CYP1B1-mediated E2 metabolism in rat EECs as compared to humans. CYP1B1-mediated metabolism of E2 can result in the formation of DNA adducts and mutagenicity which can ultimately initiate human breast and other cancers [31]. Indeed, it was reported that many tumor cells express a higher level of CYP1B1 compared to the surrounding normal tissue [32]. This is also supported by a ~15-200-fold higher excretion of estrogen-DNA adducts in urine from women with breast cancer compared to women without breast cancer [33]. It would be extremely informative for future studies to determine the effects of changes in CYP1A1/1B1 ratio in endometrial cells and link this to actual estrogen metabolite levels and/or cancer markers.

Taken together, AHR activation is related to anti-estrogenic acttions and protective against, especially, endometrial cancer. For example, smoking is associated with lower endometrial cancer risk due to AHR activation by polycyclic aromatic hydrocarbons in cigarette smoke [34]. In the present study, anti-estrogenic effects of AHR ligands in human and rat EECs were assessed using PR (both species), GREB1 (human) and HSP27 (rat) gene expression as markers of E2-responsiveness [35–38]. HSP27 gene expression was assessed in the rat EECs as, to the best of our knowledge, gene expression of GREB1 in rat uterine tissue has not been reported in literature. No anti-estrogenic effects were observed in rat EECs upon TCDD or DELAQ exposure. E2-induced PR levels in rat mammary glands were also reported not changed by TCDD [39]. Anti-estrogenic effects of TCDD have however been reported in rats *in vivo*. Chaffin et al. 1996 showed reduced circulating E2 levels in pregnant Holtzman rats after exposure to TCDD [40]. Yet, effects on uterine wet weights, an E2-dependent endpoint, were not reported [40]. In contrast, exposure to DELAQ did attenuate E2-induced expression of GREB1 in human EECs. No anti-estrogenic effects for TCDD were observed in human EECs, which may be explained by the higher potency of DELAQ to activate the AHR and induce CYP1A1 and CYP1B1 expression in human EECs compared to TCDD. As to the lack of anti-estrogenic effect in rats, this may be explained by a low basal AHR expression. Although the relative induction of CYP gene expression, as a proxy for AHR activation, is high in rat EECs as a result of a very low basal expression, the absolute CYP expression may not be high enough to induce the anti-estrogenic effects as observed in the human EECs.

5. Conclusion

This study cleary shows that primary rodent and human endometrial cells respond differently towards hormones and AHR ligands. This should be taken into account when performing human risk assessment based on rodent studies and warrants the development of human-relevant models to assess endometrial effects of xenobiotics.

6. Declaration of Interest and Funding

The authors have no conflicts of interest to declare. This study was performed for and financed by Teva Pharmaceutical Industries Ltd. with study number DS-2017-063.

7. References

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8. Supplementary Data

Table S1. Overview of primer sequences and primer-specific annealing temperatures used to determine the gene expression in endometrial epithelial cells in this study.

Encoding gene		5′	3′	Annealing Temperature (°C)
Human				
GAPDH	FP	GAAGGTGAAGGTCGGAGTCAAC		60
	RP	CAGAGTTAAAAGCAGCCCTGGT		
β-actin	FP	TTGTTACAGGAAGTCCCTTGCC		60
	RP	ATGCTATCACCTCCCCTGTGTG		
AHR	FP	ACATCACCTACGCCAGTCGC		60
	RP	TCTATGCCGCTTGGAAGGAT		
CYP1A1	FP	CAGAAGATGGTCAAGGAGCA		60
	RP	GACATTGGCGTTCTCATCC		
CYP1B1	FP	CGGCCACTATCACTGACATC		60
	RP	CTCGAGTCTGCACATCAGGA		
PR	FP	CGCGCTCTACCCTGCACTC		60
	RP	TGAATCCGGCCTCAGGTAGTT		
GREB1	FP	CAAAGAATAACCTGTTGGCCCTGC		60
	RP	GACATGCCTGCGCTCTCATACTTA		
Rat				
Arbp	FP	CCTAGAGGGTGTCCGCAATGTG		63
	RP	CAGTGGGAAGGTGTAGTCAGTCTC		
β-actin	FP	AGCGTGGCTACAGCTTCACC		60
	RP	AAGTCTAGGGCAACATAGCACAGC		
AHR	FP	TGGCTGTGATGCCAAAGGGCAG		59
	RP	AGCATGTCAGCGGCGTGGAT		
CYP1A1	FP	ATGTCCAGCTCTCAGATGATAAGGTC		60
	RP	ATCCCTGCCAATCACTGTGTCTAAC		
CYP1B1	FP	CTCATCCTCTTTACCAGATACCCG		60
	RP	GACGTATGGTAAGTTGGGTTGGTC		
PR-A+B	FP	TGGTTCCGCCACTCATCA		60
	RP	TGGTCAGCAAAGAGCTGGAAG		
HSP27	FP	CGTGGTGGAGATCACTGGCAAGC		60
	RP	CGGGCCTCGAAAGTGACCGG		

Chapter 4

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 [1]. 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 [2, 3]. 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 [3, 4]. Progesterone antagonizes the estrogenic stimulation of uterine growth by *e.g.* inhibiting estrogen receptor alpha (ERα) chromatin binding and non-transcriptional activity [5]. 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 [3].

Hypothalamic Thyrotropin-Releasing Hormone (TRH) is a direct stimulus for prolactin release via the TRH receptor in pituitary cells in both rats and humans (Figure 1). However, TRH appears to stimulate prolactin release especially when dopaminergic input is low or absent [4]. Rats exhibit a particularly sensitive feedback mechanism between prolactin secretion and estrogens, while in humans, endogenous estrogens only have a modest stimulatory effect on pituitary prolactin release [3, 4]. Rising estrogen levels drive the secretion of prolactin in rats by interacting with dopamine releasing systems in the hypothalamus [6, 7]. Dopamine, secreted from the hypothalamus, inhibits prolactin release and gene transcription from the pituitary via the dopamine receptor D2 (D2R) [4, 8]. Estrogens inhibit hypothalamic secretion of dopamine, and this subsequently results in a stimulated prolactin release from the pituitary [7, 9]. 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.

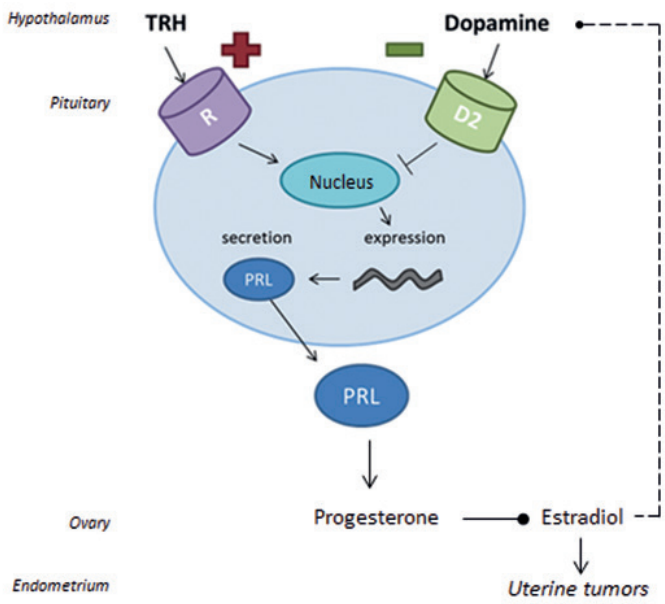

Figure 1. Schematic overview of prolactin regulation in the pituitary. TRH; Thyrotropin-Releasing Hormone, D2; Dopamine receptor D2, PRL; prolactin, R; TRH receptor.

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*. (2004) showed that the naturally occurring AHR agonist indole-3-carbinol (I3C) increased the incidence of uterine adenocarcinomas in rats [10]. Additionally, a 2-year treatment with the AHR agonist laquinimod also increased the incidence of uterine tumors in rats [11]. 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 [12-14]. 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 [15]. 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 [16]. In short, the pituitaries were minced and incubated for 45 minutes 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 minutes 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:ĐMEM 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 cells. 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 hours after seeding. All experiments were performed in triplicate. After 48 hours, the cells were exposed to thyrotropin-releasing hormone (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 48 hour 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 [17]. 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' CAGTGGGAAGGTGTAGTCAGTCTC, β-actin 5' AGCGTGGCTACAGCTTCACC 3' AGTCTAGGGCAACATAGCACAGC, Prolactin 5' CATCAATGACTGCCC CACTTC 3' CCAAACTGAGGATCAGGTTCAAA, CYP1A1 5' ATGTCCAGCTCTCAGATGATAAGGTC 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. Figure 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. Figure 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. Figure 4: N=2 for prolactin secretion and N=3 for gene expression. Figure 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 Student's t-tests were used to analyze the statistical difference of the mean between the exposed and vehicle control treatments. Symbol meaning; * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$.

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 literature [18–20]. In the primary pituitary cells, basal prolactin secretion was 454±160 ng/ml/48h. 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 (Figure 2A). Quinpirole statistically significantly inhibited prolactin secretion by 2.6-fold and prolactin gene expression by 3.6-fold in the primary pituitary cells compared to vehicle treated control cells (Figure 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/48h. 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 (Figure 2B). In contrast to the primary pituitary cells, no inhibitory effects on prolactin secretion or gene expression were observed after treatment with quinpirole alone (Figure 2B) or in combination with TRH after 24 or 48 hours (data not shown). Basal prolactin secretion in RC-4BC cells was approximately 137±27 ng/ml/48h 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 expression in the RC-4BC cells (Figure 2C).

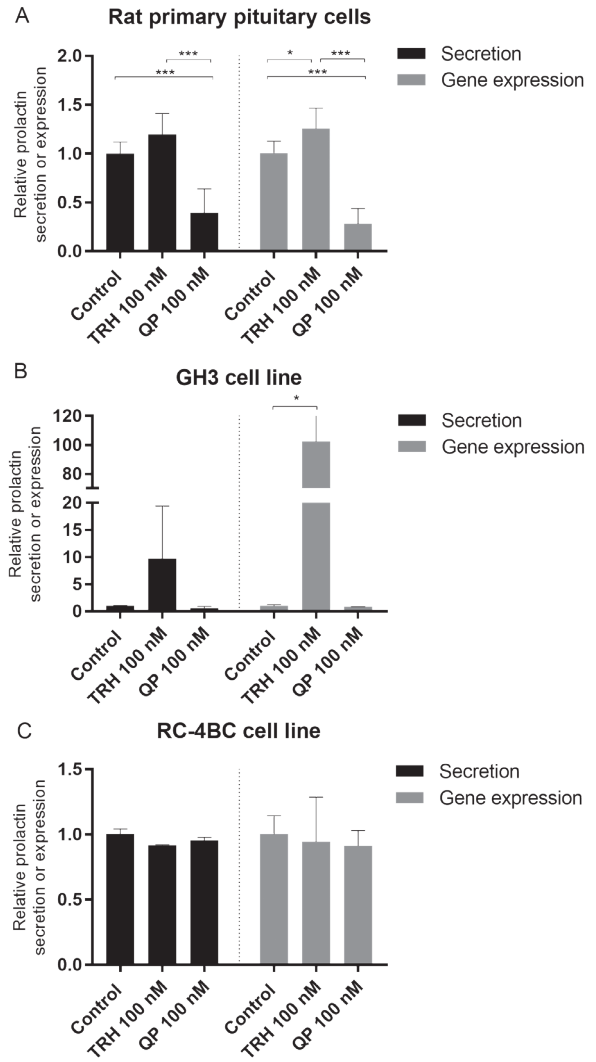

Figure 2. Prolactin secretion and gene expression in rat (A) primary pituitary cells, (B) GH3 and (C) RC-4BC cell lines. The cells were exposed to a vehicle control (0.1% DMSO), 100 nM TRH, or 100 nM quinpirole (QP) for 48 hours. Data is expressed as mean fold change (with SD) relative to the vehicle-treated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

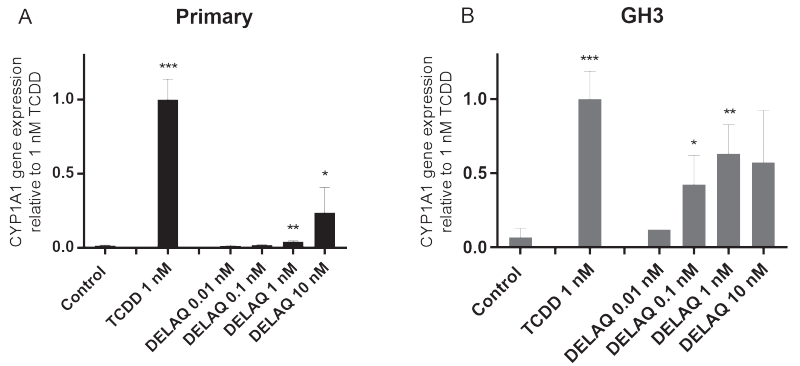

Figure 3. CYP1A1 gene expression in rat (A) pituitary primary cells (B) GH3 cells. Data is expressed as mean fold change (with SD) relative to 1 nM TCDD treated cells. *Statistically significantly different from control with (*) P≤0.05, (**) P≤0.01 and (***) P≤0.001.

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 (Figure 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 pituitary cells is already high and could not be stimulated further with TRH (Figure 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

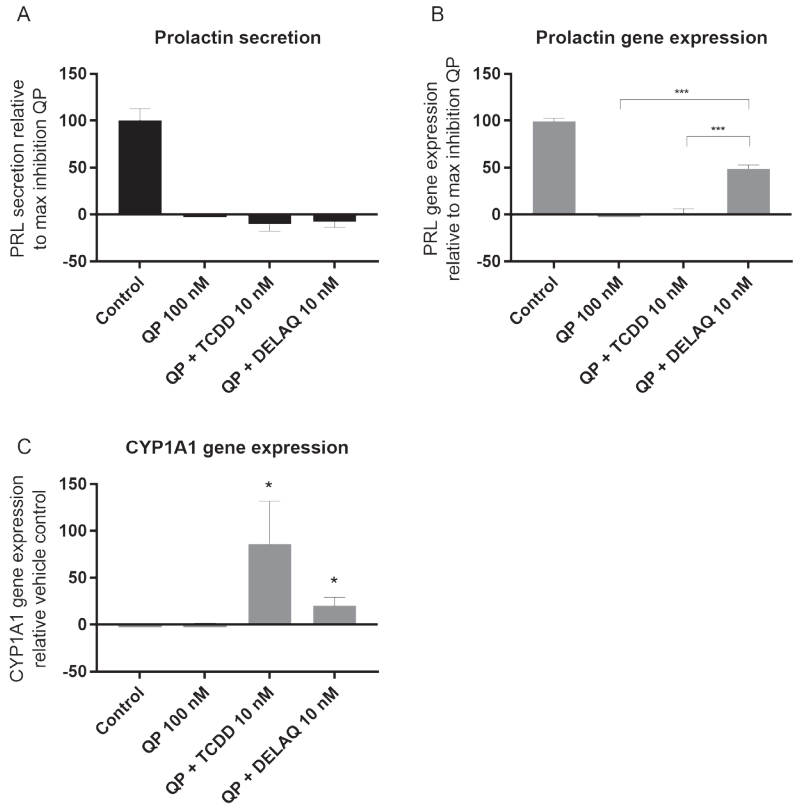

Figure 4. (A) Prolactin secretion and (B) prolactin gene expression in primary rat pituitary cells after a 48-hour exposure to 100 nM quinpirole (QP) alone or in combination with 10 nM TCDD or 10 nM DELAQ. Data (A+B) is expressed as percentage secretion or expression, where vehicle-treated control cells (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.

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 (Figure 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% (Figure 4B). In contrast to DELAQ, co-exposure with TCDD had no effect on quinpirole inhibition of prolactin gene expression.

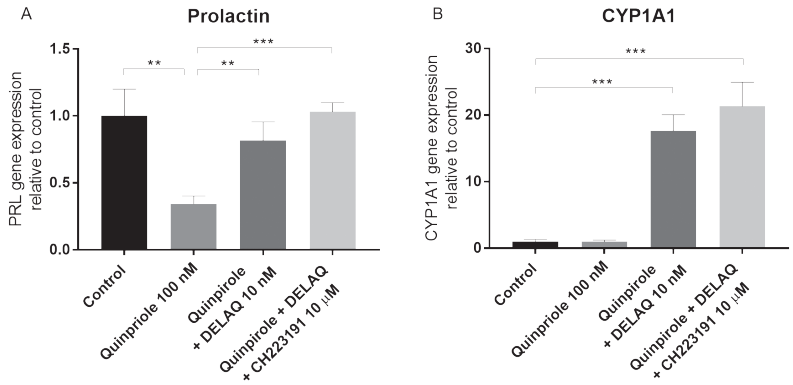

Figure 5. Fold induction in prolactin and CYP1A1 gene expression in primary pituitary cells after a 48 hour treatment with 100 nM quinpirole, 100 nM quinpirole + 10 nM DELAQ, 100 nM quinpirole + 10 nM DELAQ + 10 µM AHR antagonist CH223191. Data is expressed relative to the vehicle control (0.1% DMSO)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

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 (Figure 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 (Figure 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 (Figure 5A and 5B).

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 [21]. 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 [22]. 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 [23, 24]. The serum-free experimental conditions in our study may have contributed to the high induction of TRH-stimulated prolactin release. Brunet *et al.* (1981) 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 [25]. 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) [26]. 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 [27]. 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 [28]. 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 [29]. 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 hours 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 [30, 31]. 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 [32].

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 [15], 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 [33]. Yet, Russell *et al*. (1988) found that treatment with TCDD caused a significant decrease of serum prolactin concentrations in an *in vivo* study with rats [34]. 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 [14], leading to an increase in hypothalamic dopamine release and subsequent stronger inhibition and thus lower prolactin production in the pituitary (Figure 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*. (2010) reported on the selectively of this inhibitor to TCDD-specific action compared to other ligands, due to differential ligand binding [35]. 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 [36]. 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 [37, 38]. 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 [37, 39]. Alternative hypotheses on pituitary prolactin regulation suggest that other factors, like glucocorticoids, are involved in the regulation of prolactin secretion as well [40, 41]. 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 [42]. 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 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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8. Declaration of interest

Teva Pharmaceutical Industries Ltd. was involved in approving the study design, review of the manuscript and the decision to submit the article for publication. Utrecht University had full freedom to publish any results from this study financed by Teva.

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Chapter 5

Differential AHR-mediated anti-tumor responses in endometrial cancer cell lines Ishikawa and ECC-1

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Abstract

Aryl hydrocarbon receptor (AHR) activation can result in anti-tumorigenic effects on estrogen dependent tumor cells, such as endometrial cells, (partly) via inhibitory cross-talk with the estrogen receptor (ER). It is hypothesized that the anti-tumorigenic properties of plant-derived resveratrol act via the AHR. Therefore, the effects of resveratrol and its analogue tetramethoxystilbene (TMS) were assessed in the endometrial cancer cell lines Ishikawa and ECC-1.

Resveratrol inhibited tumor cell viability in both cell lines, but stronger in Ishikawa cells. Tumor cell migration was not affected by resveratrol in both of the endometrial cell lines tested. In addition, resveratrol increased the percentage of cells in the S-phase of the cell cycle in Ishikawa cells, whereas it did not affect the cell cycle in ECC-1 cells. 1 μM resveratrol activated the AHR as indicated by an induced gene expression of CYP1A1 with 1.5-fold in Ishikawa cells and 5-fold in ECC-1 cells.

Additionally, the resveratrol analogue TMS showed stronger anti-tumor activity than resveratrol in ECC-1 cells with 27% more reduction in viability and 50% more reduction in tumor cell migration compared to resveratrol. In Ishikawa cells, however, cell viability and migration were not affected by TMS. Gene expression data show that 1 μM TMS decreased CYP1A1 gene expression with 3-fold in Ishikawa cells, while it increased CYP1A1 gene expression 39-fold ECC-1 cells.

Resveratrol and TMS are considered anti-tumorigenic compounds, but did not evoke similar anti-tumor properties in the endometrial cancer cell lines Ishikawa and ECC-1. Anti-tumor properties were apparent in ECC-1 cells but lacking in Ishikawa cells for TMS. This coincided with AHR activation, suggesting that the activation of the AHR plays an important role in anti-tumorigenicity, but that this activation clearly differs between the two cell lines. In addition, AHR-ER cross-talk was only observed in the ECC-1 cells and not in the Ishikawa cells as measured by a reduced CYP1A1 gene expression after co-exposure to estrogen. These fundamental mechanistic differences between the two endometrial cancer cell lines indicate that conclusions with respect to endometrial effects and AHR ligands, are strongly dependent on the type of cell model that is being used.

1. Introduction

Endometrial cancer is the most common gynecological cancer and affects most often post-menopausal women. 80% of these endometrial tumors are hormone dependent, *i.e.* the cells depend on estrogen to proliferate [1].

The aryl hydrocarbon receptor (AHR) has been proposed as a target receptor for the treatment of hormone-dependent tumors like endometrial cancer [2–4]. The AHR is a cytoplastic ligand-dependent transcription factor that mediates a wide range of biological events in response to endogenous and exogenous compounds. When activated, this protein heterodimerizes, co-factors are recruited, and the ligand-receptor complex binds to dioxin responsive elements on DNA where it can initiate the transcription of AHR dependent genes, *e.g.* cytochrome P450 (CYP) 1A1 and 1B1 enzymes [5, 6].

AHR activation can also result in an inhibitory cross-talk with the estrogen receptor (ER), by interfering with ER-DNA binding to estrogen responsive elements on DNA that contain an overlapping dioxin response element sequence. This can result in down regulation of the ERα, inhibition of selected estrogen target genes, and increased estrogen metabolism by CYP1A1 and CYP1B1 enzymes [6, 7]. This was shown by AHR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which inhibited endometrial tumor cell growth *in vitro* [8], [9]. Additionally, smoking is considered to protect against endometrial cancer because of the activation of AHR by polycyclic aromatic hydrocarbons present in cigarette smoke [10, 11].

The naturally occurring plant hormone resveratrol is an AHR ligand, and has been studied for its anti-tumorigenic properties in a broad variety of cancer models. Resveratrol has been shown to inhibit tumor initiation and progression in various tumor cell lines *in vitro* as well as *in vivo* [12–14]. Even though these anti-tumorigenic properties have also been observed in hormone independent tumor cells, like breast cancer cell line MDA-MB-231, anti-tumor effects are generally greater in hormone dependent tumor cells, like the breast cancer cell line MCF-7 [15, 16]. This supports the notion that resveratrol may, partly, act via the AHR and subsequent inhibitory AHR-ER cross-talk, although the exact mechanism of resveratrol action remains unclear.

Structurally similar analogues of resveratrol, especially with additional hydroxy or methoxy groups or substitutions to the stilbene backbone of resveratrol, have been reported to exert even more potent anti-tumor properties in several tumor cell models, including hormone dependent breast cancer cells [12], [17]. Such analogues are assumed to be more biologically stable with a higher bioavailability and increased transport of the compound into the cell [18, 19]. However, these effects still have to be identified for endometrial tumor cell lines, considering the tissue-specific effects of AHR activation.

In the present study, the effects of resveratrol and its analogue tetramethoxystilbene (TMS) on tumor cell viability, migration, and cell cycle progression were assessed in two endometrial tumor cell lines Ishikawa and ECC-1. Accordingly, the expression of genes involved in the cell cycle and the AHR gene battery were assessed. In addition, the effects on the gene expression of the AHR gene battery between Ishikawa and ECC-1 were assessed using other AHR ligands, DELAQ and TCDD.

2. Methods

2.1. Chemicals

Resveratrol (3,4',5-trihydroxy-trans-stilbene) was obtained from Sigma-Aldrich, the Netherlands and its analogue tetramethoxystilbene (2,3',4,5'-tetramethoxy-trans-stilbene; TMS) was obtained from Enzo Life Sciences, Belgium. Tetrachlorodibenzodioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin; TCDD) was obtained from Wellington Laboratories Inc, Canada. Des-ethyl-laquinimod (DELAQ) was received from Teva Pharmaceutical Laboratories Ltd, Israel. All other chemicals used were obtained from Sigma-Aldrich, the Netherlands, unless stated otherwise.

2.2. Cell culture

The Ishikawa cell line was derived from a human endometrial adenocarcinoma and was purchased from Sigma-Aldrich, Germany. The ECC-1 cell line was also derived from a human endometrial adenocarcinoma and purchased from ATCC-LGC standards. The Ishikawa cells were cultured in MEM culture medium (Invitrogen) supplemented with 1% penicillin/streptomycin (Invitrogen), 5% fetal bovine serum (FBS; Invitrogen), 1% glutamine (Invitrogen) and 1% non-essential amino acids (Invitrogen). The ECC-1 cells were cultured in RMPI culture medium without phenol red (Invitrogen) and supplemented with 1% penicillin/streptomycin and 5% FBS. The cells were maintained in 75 cm^2 culture flasks in a humified atmosphere with 5% CO_2 at 37 °C and subcultured twice a week at a cell density of 70%.

2.3. Cell viability

The Ishikawa cells were seeded with a density of $5*10^4$ cells/well on a 48-well plate and the ECC-1 cells with a density of $8*10^4$ cells/well. After 24 hours, the cells were exposed to the test compounds in a range of 0-30 µM for 3 days. 0.1% DMSO was used as a vehicle control. The viability of the cells was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. 1 mg/ml MTT was added to each well and incubated for 1.5 hour, after which isopropanol was used to lyse the cells. Absorbance was measured at 595 nm as a proxy for the number of cells. This experiment was performed four times in triplicate for the Ishikawa cells and twice in triplicate for the ECC-1 cells.

2.4. Migration assay

A wound healing assay was performed to assess the migration of Ishikawa and ECC-1 cells in the presence of resveratrol and TMS. Ishikawa cells were seeded at a density of $3*10^5$ cells/well in a 12-well plate, ECC-1 cells were seeded at a density of $5*10^5$ cells/well. After 48 hours, a scratch was made into the monolayer of cells using a 200 µl pipette tip. The wells were rinsed to remove the unattached cells and the culture medium was replaced with culture medium containing either 0.1% DMSO, 10 µM resveratrol or 10 µM TMS. Snapshots of the wound area were taken at 0 hours, 24 hours and 48 hours and wound surface area was analyzed using ImageJ software [20]. This experiment was performed three times, in triplicate.

2.5. Cell cycle assay

The Ishikawa cells were seeded with a density of $3*10^5$ cells/well on a 12-wells plate and the ECC-1 cells were seeded with a density of $4*10^5$ cells/well. The cells were exposed to either 0.1% DMSO, 3 µM resveratrol, 3 µM TMS or 1 µM 2-Methoxyestradiol (2-MeOE2) for 6, 12 or 24 hours and fixed with 70% ethanol until further analysis. Cell cycle analysis was performed by staining the cells with propidium-iodide (PI). PI content was measured with the use of flow cytometry (C6 Accuri) to analyze the distribution of the cells in each phase of the cell cycle. Multi-nucleate or otherwise aberrant cells

were excluded from the analysis [21]. This experiment was performed three times, in triplicate.

2.6. mRNA isolation and RT-qPCR

The Ishikawa cells were seeded with a density of $1*10^5$ cells/well on a 12-well plate and the ECC-1 cells were seeded with a density of $2*10^5$ cells/well. The cells were exposed for 48 hours to a concentration of 1 µM (resveratrol and TMS) or 1 nM (TCDD and DELAQ) of the test compounds, with or without 0.1 nM estradiol (E2). Vehicle controls were treated with 0.1% DMSO, or 0.1% DMSO + 0.1% ethanol. Tri-reagent was added to the cells to obtain the mRNA. This was isolated from the cells using the chloroform-phenol extraction method [20]. RNA concentration and purity of the samples were determined using a spectrophotometer at an absorbance wavelength of 260/280 and 230/260 nm. Samples were diluted and subsequently stored at -80 °C until further analysis.

Complementary DNA (cDNA) was synthesized with 1 µg RNA using the iScript cDNA Synthesis Kit, according to the manufacturer's instruction, using (Bio-Rad). 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 using blue shell 96-well plates (Bio-Rad) in the BioRad CFX96 C1000 thermal cycler. A reaction mix was prepared containing 7.5 µl of IQ SYBR Green Supermix (Bio-Rad, 170-8887), 0.6 µl forward primer, 0.6 µl reverse primer and 0.6 µl water. The mixture was heated initially at 95 ˚C for 3 minutes, followed by 40 cycles with denaturation at 95 ˚C for 15 seconds and annealing/extension for 45 seconds. Oligonucleotide sequences and annealing temperatures of the primers used are displayed in Table 1. Gene expression was determined using the ΔΔCq method of relative quantification where β-actin and GAPDH were used as housekeeping genes to normalize the gene expression. This experiment was performed once, in triplicate.

Table 1. Primer sequences and annealing temperatures used in real-time PCR analysis.

Gene name	Forward primer 5'→'3	Reversed primer 3'→'5	Annealing temperature (in ˚C)
AHR	ACATCACCTACGCCAGTCGC	TCTATGCCGCTTGGAAGGAT	60
Cyclin D1	GCCGAGAAGCTGTGCATCTAC	TGAGCTTGTTCACCAGGAGC	55
Cyclin E1	CAAACTCAACGTGCAAGCCTC	GCCCAGCTCAGTACAGGCAG	60
CYP1A1	TGATTGGCAGGTCACGGCGG	TGTTGTGCTGTGGGGGATGGTG	60
CYP1B1	CGGCCACTATCACTGACATC	CTCGAGTCTGCACATCAGGA	60
CDK2	ATCCGCCTGGACACTGAG	TCCGCTTGTTAGGGTCGT	65
P21	GGAAGACCATGTGGACCTGT	GGCGTTTGGAGTGGTAGA AA	60
P27	GCCCTCCCCAGTCTCTCTTA	TCAAAACTCCCAAGCACCTC	60
GAPDH	GAAGGTGAAGGTCGGAGTCAAC	CAGAGTTAAAAGCAGCCCTGGT	60
β-actin	TTGTTACAGGAAGTCCCTTGCC	ATGCTATCACCTCCCCTGTGTG	60

2.7. Data analysis

All data is expressed as mean + standard deviation of independent experiments (N) that were performed in triplicate. GraphPad Prism 7 was used to analyze and display the data. Student's T-tests were performed to determine statistical differences between the vehicle control and treatments in the viability assay, migration assay, cell cycle assay and gene expression analysis.

3. Results

3.1. Effects of resveratrol and TMS on endometrial tumor cell viability

The human endometrial cell lines Ishikawa and ECC-1 were exposed to resveratrol and TMS to assess their effect on tumor cell viability. The cells were exposed for 3 days to a concentration range up to 30 µM. Tumor cell viability decreased by 60% after exposure to 30 µM resveratrol in the Ishikawa cells, and it decreased by 30% in the ECC-1 cells (Figure 1). After exposure to TMS, the tumor cell viability was not affected in Ishikawa cells, but decreased by 57% in the ECC-1 cells at a concentration of 30 µM. Therefore, all studies were performed with a maximum test compound concentration of 10 µM.

3.2. Effects of resveratrol and TMS on endometrial tumor cell migration

A wound healing assay was performed to assess the effects of 10 µM resveratrol and 10 µM TMS on the migration potential of Ishikawa and ECC-1 endometrial tumor cells. A scratch was created in a monolayer of cells and the wound surface was measured as a marker for cell migration. After 48 hours, the wound surface area in the vehicle-treated control cells was 60% of the initial wound area in Ishikawa cells, and 45% in ECC-1 cells. Resveratrol did not affect the migration of tumor cells in neither Ishikawa nor ECC-1 cells. TMS did not affect the migration of Ishikawa cells, but statistically significantly inhibited tumor cell migration in ECC-1 cells with 50% compared to the vehicle-treated control cells after 48 hours (Figure 2).

3.3. Effect of resveratrol and TMS on cell cycle status

The cell cycle of Ishikawa cells is shorter than that of ECC-1 cells, based on the observed cell doubling times. Therefore, cell cycle status was determined after 6 and 12 hours for Ishikawa, and 12 and 24 hours for ECC-1 cells. The cell cycle status of both Ishikawa and ECC-1 cells was analyzed after exposure to 10 µM resveratrol, 10 µM TMS, and 1 µM 2-MeOE2, a known inducer of cell cycle arrest in the G2/M phase of the cycle [12].

In Ishikawa cells, a higher percentage of the cells (47%) were observed in the G1 phase of the cell cycle after a 6-hour treatment with resveratrol compared to the vehicle treatment (35%) (Figure 3A). This was accompanied by a lower percentage of cells in the G2/M phase (31%), compared to the vehicle treatment (47%). Treatment with 2-MeOE2, decreased the number of Ishikawa cells in the G1 phase (21%) and increased the number of cells in the G2/M phase (62%). After a 12-hour treatment, however, no apparent effect of resveratrol was observed on the number of cells in the G1 or G2/M phase of the cell cycle (Figure 3B). Treatment with 2-MeOE2 decreased the number of Ishikawa cells in the G1-phase (from 51% to 14%) and increased the cells in the G2/M-phase of the cell cycle (from 33% to 70%). TMS did not affect the cell cycle of Ishikawa cells after a 6- nor 12-hour treatment.

None of the compounds tested statistically significantly affected the cell cycle status of ECC-1 cells after 12 nor 24 hours (Figure 3C and D).

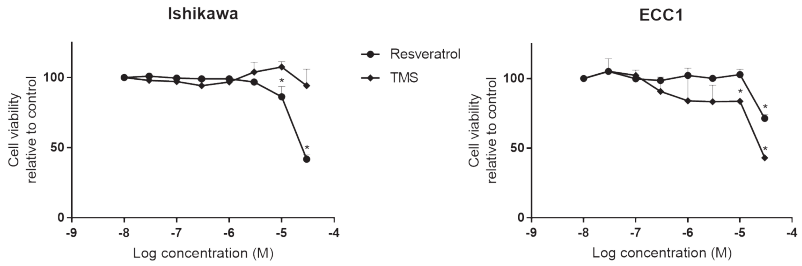

Figure 1. Cell viability assay in Ishikawa and ECC-1 cells. Cells were exposed to a concentration range (0 to 30 µM) of resveratrol and its analogue tetramethoxystilbene (TMS). Data is expressed relative to the vehicle treated control cells (0.1% DMSO) that was set to 100%, and expressed as mean ± standard deviation. *Statistically significantly different from vehicle-treated (0.1% DMSO) control cells with *P≤0.05.

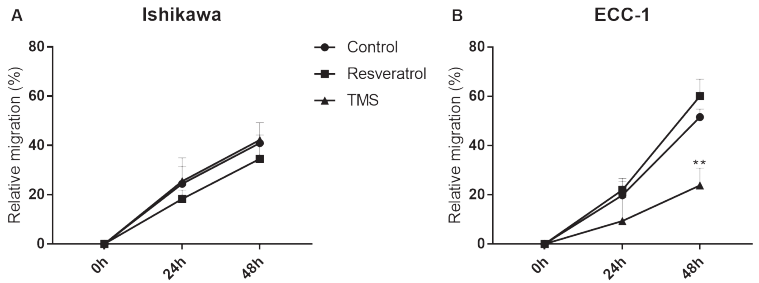

Figure 2. Relative migration of Ishikawa (A) and ECC-1 (B) cells in a wound healing assay. Cells were exposed to 0.1% DMSO (vehicle control), 10 µM resveratrol and 10 µM TMS for up to 48 hours. Data is represented as mean relative migration compared to the wound at 0 hours for every individual treatment. *Statistically significantly different from vehicle-treated control cells at 48 hours ** P ≤ 0.01

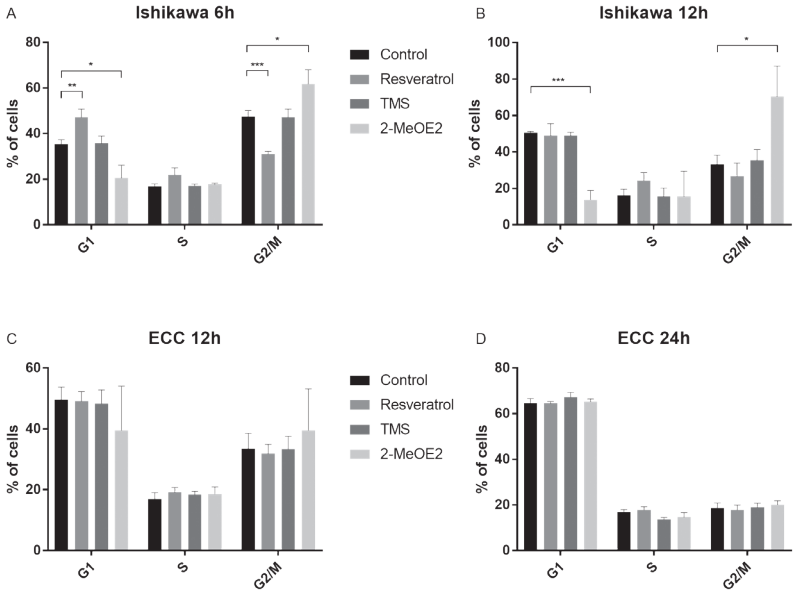

Figure 3. Analysis of the cell cycle status of Ishikawa and ECC-1 cells. Cells were exposed to 10 µM resveratrol, tetramethoxystilbene (TMS) and 1 µM 2-methoxyestradiol (2-MeOE2) for 6 hours (Ishikawa), 12 hours (Ishikawa and ECC-1) or 24 hours (ECC-1). Bars represent the percentage of cells distributed over each phase of the cell cycle and are expressed as mean ± standard deviation. *Statistically significantly different from vehicle-treated (0.1% DMSO) control cells with * P≤0.05, ** P≤0.01 and *** P≤0.001.

3.4. Effect of AHR ligands on gene expression

Cell cycle gene battery

E2 did not affect cell cycle related genes cyclin D, cyclin E, p21 and p27 gene expression in Ishikawa cells. Gene expression of these genes were also not affected by resveratrol. Only p27 gene expression increased 1.3-fold after exposure to TMS in Ishikawa cells, and 1.5-fold after co-exposure to TMS and E2.

E2 did also not affect cyclin D, cyclin E, p21 and p27 gene expression in ECC-1 cells. Also, no significant effect on these genes was observed after treatment with resveratrol. TMS however decreased cyclin D and cyclin E gene expression

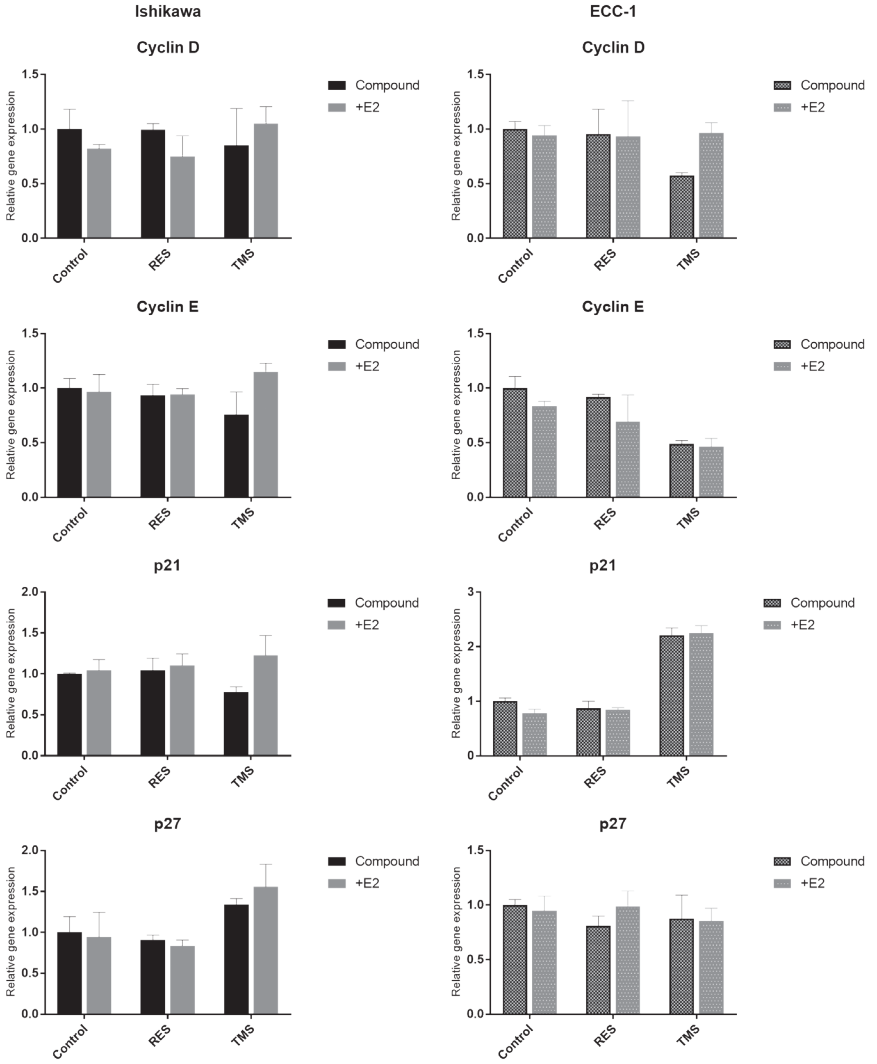

Figure 4. Gene expression of cell cycle gene battery in Ishikawa (left) and ECC-1 (right) cells. Cells were exposed for 48 hours to 1 µM resveratrol or 1 µM TMS, with or without 0.1 nM E2, or treated with the vehicle control (0.1% DMSO or 0.1% DMSO + 0.1% ethanol). Data is expressed relative to the vehicle-treated control treatment.

with 0.6-fold and 0.4-fold respectively in ECC-1 cells. TMS increased p21 gene expression 2.2-fold upon exposure in ECC-1 cells. E2 attenuated the effect of TMS on cyclin D inhibition.

AHR gene battery

In addition to resveratrol and TMS, the cell lines were exposed to 1 nM TCDD and DELAQ to further assess the differences between the cell lines with regards to AHR activation. TCDD and DELAQ were previously shown to affect AHR signaling in primary endometrial cells with different potencies [22]. Again, Ishikawa and ECC-1 cells were also exposed to E2 to assess the potential effects of the AHR ligands on ER-AHR cross-talk in the cell lines.

Neither E2 or any of the AHR activating compounds changed AHR gene expression in Ishikawa cells upon exposure (Figure 5A).

Both CYP1A1 and CYP1B1 gene expression increased 1.5-fold after exposure to 1 μM resveratrol in Ishikawa cells, with no significant effect of co-exposure to E2 (Figure 5B and C). However, CYP1A1 and CYP1B1 gene expression decreased 3-fold and 1.8-fold respectively after exposure to 1 μM TMS, compared to the vehicle control and with no significant effect of co-exposure to E2. CYP1A1 gene expression increased 7-fold after exposure to DELAQ and 35-fold after exposure to TCDD, with no significant effect of co-exposure to E2. CYP1B1 gene expression increased 6-fold after exposure to DELAQ, and 12-fold when Ishikawa cells were co-exposed to DELAQ and E2. CYP1B1 gene expression increased 17-fold after exposure to TCDD, with no significant effect of co-exposure to E2.

In ECC-1 cells, both E2 and TMS decreased AHR gene expression by 1.6-fold. The other AHR ligands did not affect AHR gene expression. In combination with E2 however, DELAQ and TCDD increased the gene expression of the AHR (Figure 5D).

CYP1A1 and CYP1B1 gene expression increased 5-fold and 1.3-fold, respectively, after exposure to 1 μM resveratrol in the ECC-1 cells (Figure 5E and F). Co-exposure with E2 resulted in an attenuation of CYP1A1 and CYP1B1 gene expression induced by resveratrol, with an increased gene expression of 3.1-fold and 1.3-fold, respectively, compared to the control treatment. CYP1A1 and CYP1B1 gene expression respectively increased 39-fold and 3-fold after exposure to 1 μM TMS. Co-exposure with TMS and E2 did not affect CYP expression as was observed with resveratrol and E2.

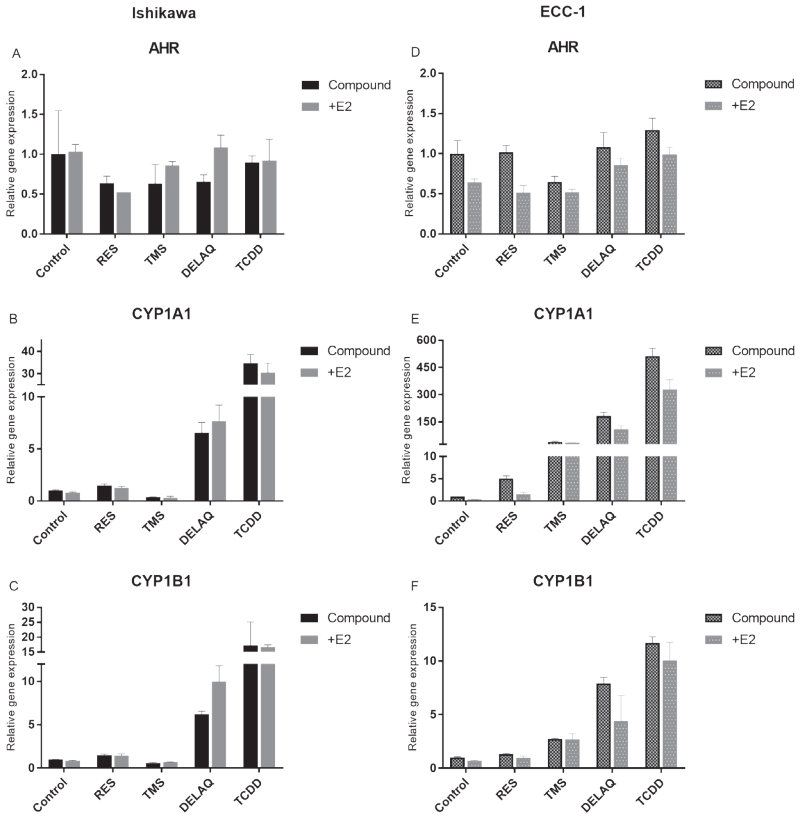

Figure 5. Gene expression of AHR gene battery in endometrial cell lines Ishikawa (left) and ECC-1 (right). Cells were exposed for 48 hours to 1 μM (resveratrol and TMS) or 1 nM (TCDD and DELAQ) of the test compound with or without 0.1 nM E2, or treated with the vehicle (0.1% DMSO or 0.1% DMSO+0.1% ethanol). Data is expressed relative to the vehicle-treated control treatment.

CYP1A1 gene expression increased 180-fold after exposure of ECC-1 cells to DELAQ and 510-fold after exposure to TCDD compared to the control treatment. Co-exposure of DELAQ and TCDD with E2 resulted in a respective 300-fold and 900-fold increase of CYP1A1 compared to the cells treated with E2 alone. However, when compared to the normalized expression of CYP1A1 after treatment with only DELAQ and TCDD, this gene expression was reduced with 40% and 35%, respectively (Figure 5E).

CYP1B1 gene expression increased 8-fold after exposure to DELAQ and 12-fold after exposure to TCDD compared to the control treatment. Co-exposure of DELAQ and TCDD with E2 resulted in a respective 6-fold and 15-fold increase of CYP1B1 expression compared to the E2-treated control cells. When compared to the normalized expression of CYP1B1 after treatment with only DELAQ and TCDD, this gene expression was reduced with 45% and 15% respectively (Figure 5F).

4. Discussion

The anti-tumorigenic effects of resveratrol and its methoxylated analogue tetramethoxystilbene (TMS) were assessed in the endometrial tumor cell lines Ishikawa and ECC-1. In ECC-1 cells, TMS displayed stronger anti-tumorigenic properties (*i.c*. reduced tumor cell viability and migration) than resveratrol, while in Ishikawa cells, only resveratrol displayed anti-tumorigenicity and no effect of TMS was observed. The observed effects appear to coincide with the induction of AHR-dependent genes, suggesting that the anti-tumorigenic are, partly, mediated by activation of the AHR.

Resveratrol did not affect cell migration in Ishikawa cells, but it inhibited tumor cell viability and changed the cell cycle status of the Ishikawa cells. An increase in the S-phase of the cell cycle distribution was only observed in the Ishikawa cells after treatment with resveratrol. An earlier study also reported such an S-phase arrest as a result of treatment with 300 µM resveratrol in HCE7 human esophageal squamous carcinoma and HL60 human promyelocytic leukemia cell lines [23]. It should be noted that the non-statistically significant increase in the S-phase of the cell cycle observed in our experiments was observed using a concentration which was up to 2 orders of magnitude lower than the concentration used in the experiments of Joe *et al*. (2002). The same authors also report a decrease in gene expression of cyclin D after exposure to this high concentration of resveratrol. However no effect on the gene expression of cyclin D was observed in the Ishikawa cells. Again, this may be explained by the (much) lower concentration resveratrol that was used in our study.

p27 is regarded as a cyclin-dependent kinase inhibitor of G1-phase transition to the S-phase, therefore, an effect on p27 gene expression would be expected to lead to a cell cycle arrest in the G1 phase. Remarkably, TMS did not affect tumor cell viability, migration nor cell cycle status of Ishikawa cells, despite the increase in p27 gene expression after treatment with TMS. Conversely, high p27 expression is also associated with poorer prognosis in endometrial tumor cells *in vivo,* as it is linked to higher proliferation [24]. This paradoxical high expression of p27 after TMS treatment corresponds to the continued proliferation that was observed in the Ishikawa cells treated with TMS. This may explain the lack of anti-tumor properties, *i.c.* on tumor cell viability and migration, in Ishikawa after TMS treatment which were observed in ECC-1 cells.

TMS showed more potent anti-tumor properties compared to resveratrol in the ECC-1 cell line. This is similar to what is described for breast tumor cell lines and is possibly related to a higher bioavailability or compound stability as a result of the methoxy-groups present on the stilbene backbone of TMS [12, 18]. However, no inhibitory effect of 10 µM resveratrol or TMS on the cell cycle status of ECC-1 cells was observed. On the gene expression level however, 1 µM TMS did affect the expression of cell cycle related genes p21, cyclin D and cyclin E. p21 gene expression also increased while cyclin D and cyclin E gene expression also decreased after exposure to up to 100 µM resveratrol in MCF-7 and MDA-MB-231 breast cancer cells [15]. Possibly, the concentrations that were used in these experiments did affect the cell cycle related genes, but were too low to evoke a clear effect on the cell cycle distribution. Also considering that the cell cycle of ECC-1 cells, *i.e*. the cell doubling time is considerably longer than other cell lines such as Ishikawa and MCF-7, in the future it may be more appropriate to use other time points such as 36 or 48 hours in addition to a higher dose to evoke effects on cell cycle distribution.

It was suggested that the anti-tumor properties of resveratrol and its analogue TMS can be mediated by AHR activation. In our experiments, the gene expression of AHR-dependent genes CYP1A1 and CYP1B1 was used as a proxy for AHR activation in the cell lines. TMS increased CYP1A1 and CYP1B1 gene expression more than resveratrol did in ECC-1 cells, and corresponds to the observed stronger anti-tumorigenic properties in this cell line. This supports the notion that the observed anti-tumor effects of resveratrol and TMS are indeed AHR-dependent.

In contrast, TMS decreased CYP1A1 and CYP1B1 gene expression in Ishikawa cells, while resveratrol moderately increased CYP1A1 and CYP1B1 expression. The lack of any AHR activation by TMS in the Ishikawa cells may explain

the absence of anti-tumorigenic properties in these cells. It remains to be established why TMS does not display AHR activating properties in Ishikawa cells in contrast to the ECC-1 cells. It would be useful to determine the effects of resveratrol and/or analogues in human primary endometrial tissue, as this may represent the human *in vivo* situation better. Based on those outcomes, a statement could be made about the relevance and applicability of the Ishikawa and ECC-1 cell lines for endometrial risk assessment of AHR ligands.

In addition to inhibiting estrogen-signaling, co-exposure with E2 and AHR ligands has been reported to decrease AHR gene battery gene expression in several cancer cell lines as a result of an apparent two-way interaction in AHR-ER cross-talk [25]. Both receptors require similar co-factors upon their activation and as a result of squelching for these co-factors, simultaneous activation can result in lower signaling of both receptors. A lower induction of CYP1A1 and CYP1B1 was observed upon co-exposure to E2 compared to the vehicle treated control ECC-1 cells in this study, but not in the Ishikawa cells. Such AHR-ER cross-talk has also been reported for ECC-1 cells, where estrogen treatment reduced the TCDD-induced gene expression of CYP1A1, and TCDD treatment reduced the expression of the ER [8, 26, 27]. As expression of CYP1B1 can result in an increase of genotoxic estrogen metabolites, a reduction of this expression in the ECC-1 cells can be considered an anti-tumorigenic effect. A downregulation of ERα is also considered an anti-tumorigenic effect as ERα plays a role in the proliferation of endometrial cells. Nonetheless, van Ede *et al.* (2010) reported no statistically significant change in the ER status of ECC-1 cells after exposure to TCDD [28]. Similar to our observations, a lack of such AHR-ER cross-talk has been reported in Ishikawa cells [9]. On the contrary, an increase in CYP1B1 expression was observed in Ishikawa cells after co-exposure with E2 and AHR ligand DELAQ. This increase was not observed for CYP1A1, or the other AHR ligands. Sissung *et al.* (2006) describe an estrogen responsive element (ERE) in the CYP1B1 promotor, which explains the observed increase in CYP1B1 expression upon co-exposure to E2 [29]. This stimulatory effect of E2 on CYP1B1 in Ishikawa cells is in contrast with the anti-estrogenic effect that is observed in the ECC-1 cells. Possibly, the ER status and/or ERα/ERβ ratio differs between the endometrial cell lines, whether or not as a result of interference with the progesterone receptor [9]. As ERα is involved in this regulation of CYP1B1, this can explain the differential effect of E2 that is observed with regards to CYP regulation in the two endometrial cell lines. As ERβ has been shown to be involved in inhibition of proliferation, invasion and tumor formation, this can also explain the different effect between the cell lines [11, 30, 31].

Alternatively, the basal expression of the AHR may be low(er) in Ishikawa cells compared to the ECC-1 cells, as indicated by the higher Ct values in the RT-qPCR. As a result, squelching for co-factors upon ER activation may not be limiting to AHR activation in these cells [32]. This can explain the apparent lack of AHR-ER cross-talk in Ishikawa cells, in contrast to the ECC-1 cells. Lastly, hormone receptor status as well as AHR responsiveness can vary within cell lines as a result of growth conditions and cell passage [9]. Further research is warranted to identify the root of these differences in AHR-ER cross-talk between the two endometrial cell lines.

5. Conclusion

Although both endometrial adenocarcinoma cell lines, there are significant differences observed in anti-tumor effects of AHR ligands between Ishikawa and ECC-1 cells. This can have consequences for the translation of *in vitro* studies using these cell lines for the *in vivo* situation, especially with regards to AHR activation and anti-tumor responses. Future studies should take into consideration the apparent lack of an AHR-ER inhibitory pathway in Ishikawa when using this cell line. This also suggests that the Ishikawa may not be suitable to support conclusions with respect to endometrial cancer effects and environmental exposures using AHR ligands.

6. References

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Chapter 6

Anti-tumor properties of methoxylated analogues of resveratrol in malignant MCF-7 but not in non-tumorigenic MCF-10A mammary epithelial cell lines.

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Abstract

Resveratrol is a plant-derived polyphenol that is known for its anti-inflammatory and anti-tumorigenic properties in *in vitro* and *in vivo* models. Recent studies show that some resveratrol analogues might be more potent anti-tumor agents, which may partly be attributed to their ability to activate the aryl hydrocarbon receptor (AHR). Here, the anti-tumorigenic properties of resveratrol and structural analogues oxyresveratrol, pinostilbene, pterostilbene and tetramethoxystilbene (TMS) were studied *in vitro*, using in the malignant human MCF-7 breast cancer cell line and non-tumorigenic breast epithelial cell line MCF-10A.

Cell viability and migration assays showed that methoxylated analogues of resveratrol are more potent anti-tumorigenic compounds than resveratrol and its hydroxylated analogue oxyresveratrol, with 2,3',4,5'-tetramethoxy-*trans*-stilbene (TMS) being the most potent compound. TMS decreased MCF-7 tumor cell viability with 50% at 3.6 μM and inhibited migration with 37.5 ± 14.8% at 3 μM. In addition, TMS activated the AHR more potently (EC50 in a reporter gene assay 2.0 μM) and induced AHR-mediated induction of cytochrome P450 1A1 (CYP1A1) activity (EC50 value of 0.7 μM) more than resveratrol and the other analogues tested. Cell cycle analysis showed that TMS induced a shift in cell cycle status from the G1 to the G2/M phase causing a cell cycle arrest in the MCF-7 cells, while no effect of TMS was observed in the non-tumorigenic MCF-10A mammary epithelial cell line. Gene expression analysis showed that 3 μM TMS increased gene expression of CYP1A1 (289-fold), CYP1B1 (5-fold) and Nqo1 (2-fold), and decreased gene expression of IL-8 (3-fold) in MCF-7 cells. In MCF-10A cells, 10 μM TMS also increased gene expression of CYP1A1 (5-fold) and CYP1B1 (2-fold), but decreased gene expression of Nqo1 (1.4-fold) in contrast to MCF-7 cells.

TMS displays more potent anti-tumorigenic properties and activates the AHR more effectively than resveratrol. In addition, this is the first study to show that TMS, but not resveratrol, selectively inhibits the cell cycle of breast tumor cells and not the non-tumorigenic cells. Our study provides more insight in the anti-tumor properties of the methoxylated analogues of resveratrol in breast cells *in vitro*.

1. Introduction

Resveratrol is a phytoalexin naturally occurring in grapes, peanuts and pines, and is present in many dietary products. It was proposed to be an anti-inflammatory agent in the late 90s, and has received great scientific attention since [1]. Next to its anti-inflammatory properties, resveratrol has been shown to possess anti-cancer properties and act as cancer chemopreventive (*i.c.* inhibit tumor initiation) and anti-tumor agent (*i.c.* inhibit tumor progression) in various *in vitro* and *in vivo* cancer models, including the human breast cancer cell line MCF-7 [2-4].The exact anti-cancer mechanism of action of resveratrol remains unclear, but there are several studies that suggest that part of resveratrol action is mediated via the aryl hydrocarbon receptor (AHR) [5, 6].

The AHR is a cytoplasmic ligand-dependent transcription factor that mediates a wide range of biological and toxic events in response to endogenous and exogenous ligands. When activated by ligand binding, the receptor translocates to the nucleus and initiates the transcription of, among others, cytochrome P450 (CYP) 1A1 and CYP1B1 expression [7]. These phase I metabolizing enzymes are expressed in many tissues, including cancer cells. CYP enzymes metabolize both exogenous and endogenous substrates, including the endogenous steroid hormone estradiol (E2). Here, especially CYP1B1-mediated metabolism of estrogens is of concern, as this may lead to genotoxic estrogen metabolites [8]. CYP1B1 is also able to metabolize resveratrol to piceatannol, which is known for its anti-cancer properties from *in vitro* and *in vivo* studies and may partly explain the potency of resveratrol to protect cells against tumorigenic effects [5, 9]. In addition to the regulation of some CYP enzymes, activation of the AHR is involved in a wide range of cellular responses. These include, among others, the modulation of tumor suppressing or promoting factors such as interaction with the Nrf2 pathway [10, 11] and modulation of interleukin-8 (IL-8) expression [12, 13].

Many studies have reported that AHR activators can inhibit the growth of a wide variety of tumor cells, like breast and endometrial tumor cells [14–16]. Although the anti-tumorigenic properties of resveratrol have also been observed in hormone independent breast tumor cells, these effects are generally greater in hormone dependent breast tumor cells. Such tumor cells express various steroid receptors and tumor progression can be stimulated by E2 and progesterone (P4). AHR ligands have been shown to modulate the function of

steroid receptors and inhibit the tumorigenic response to steroid hormones. This cross-talk between the AHR and ER responsive pathways indicate another mechanism via which resveratrol can exert its anti-tumorigenic action in hormone-responsive tumor cells [5, 6]. The AHR is therefore often suggested to be a potential target for cancer treatment, as it is higher expressed in many tumors compared to normal tissues [17–20].

Although resveratrol itself displays anti-cancer properties, adding hydroxyl or methoxy groups or substitutions to the stilbene backbone of resveratrol may result in analogues that may be more active than resveratrol. These methoxylated analogues are assumed to be more biologically stable with a higher bioavailability and an increased transport of the compound into the cell [21–23]. Indeed, several *in vitro* and *in vivo* studies have indicated that other synthetic and natural derivatives of resveratrol can have a higher anti-cancer potency than resveratrol in various cancer models [24–33].

We studied the anti-tumor properties of resveratrol and several analogues with varying hydroxy and/or methoxy substitutions in the human breast cancer cell line MCF-7 (Figure 1). The effects of these compounds were studied on tumor cell viability and migration. In addition, we assessed the potency of these compounds to activate the AHR using a reporter gene assay and subsequent CYP1A1 enzyme activity. The anti-tumor properties of resveratrol and its most potent analogue tetramethoxystilbene (TMS) were further studied in human breast cancer cells (MCF-7) and compared with effects in non-tumorigenic (MCF-10A) mammary epithelial cells. For that, cell cycle status and gene expression patterns of selected genes that are linked to carcinogenicity and AHR activation were assessed in both cell lines.

2. Material and methods

2.1. Chemicals

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), and its derivatives oxyresveratrol (2,3′,4,5′-tetrahydroxy-*trans*-stilbene), pinostilbene (3,4′-dihydroxy-5-methoxy-*trans*-stilbene hydrate) and pterostilbene (3,5-dimethoxy-4′hydroxy-*trans*-stilbene) were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands. Tetramethoxystilbene (2,3′,4,5′-tetramethoxy-*trans*-stilbene) was obtained from Enzo Life Sciences, Antwerp, Belgium. All other chemicals used were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands unless stated otherwise.

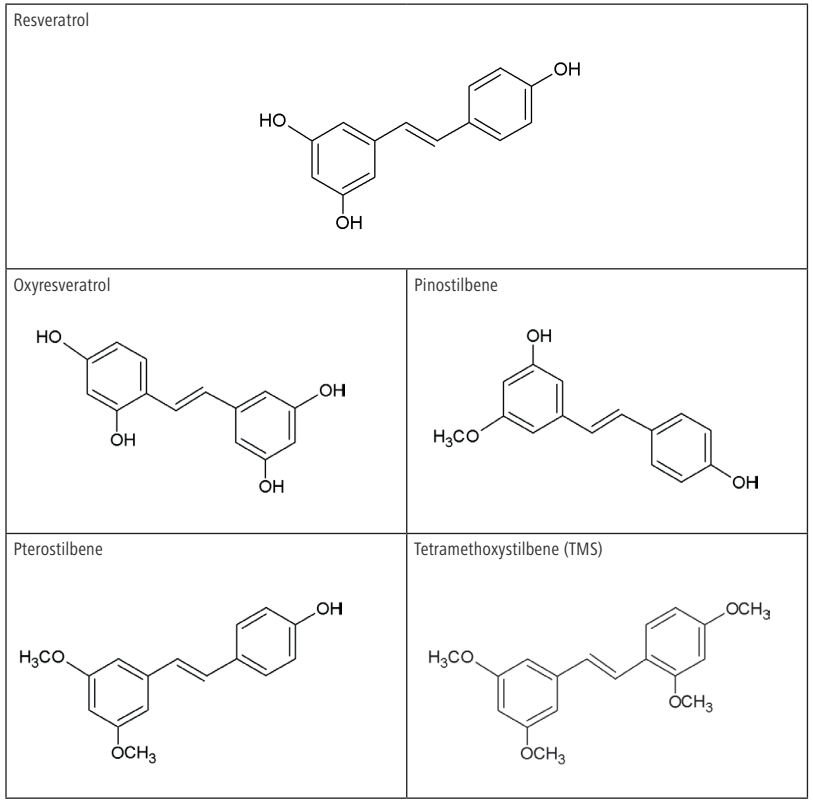

Figure 1. Structure of resveratrol and four of its analogues used in this study

2.2. Cell culture

Human breast carcinoma MCF-7 and human breast epithelial MCF-10A cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) The MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) supplemented with 5% fetal bovine serum (Life Technologies) and 0.01 mg/mL insulin (Life Technologies). The MCF-10A cells

were cultured in Dulbecco's Modified Eagle's Medium/ Ham's F12 Medium (Life Technologies) (1:1) supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 0.01 mg/mL insulin and 500 ng/mL hydrocortisone (Life Technologies). Both culture media contained 100 U/mL penicillin and 100 µg/mL streptomycin (pen/strep). The cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C, and subcultured twice a week at a cell density of 70-80%.

2.3. Cell viability

3 days prior to the experiment, the MCF-7 culture medium was replaced with assay medium that contained 5% charcoal-stripped FBS (Hyclone) to remove hormones from the medium. MCF-7 cells were seeded at a density of 8000 cells/well on a 96-wells plate. After a 2-day incubation, the cells were exposed to ranges of resveratrol and analogues in concentrations up to 30 µM. 0.1% DMSO was used as a vehicle control. Medium containing the compounds was refreshed after 4 days and the experiment was finished after a total of 10 days. The viability of the cells was determined by using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. 1 mg/ml MTT was added to each well and incubated for 30 min to allow the cells to take up the MTT. Isopropanol was used to lysate the cells and absorbance could be measured at 595 nm. The viability assay was repeated 3 times with a replicate of 3 in each experiment.

2.4. Migration assay

A wound healing assay was performed to assess the migration of MCF-7 cells in the presence of resveratrol and analogues. MCF-7 cells were seeded at a density of $1.5*10^6$ cells/well in a 12-wells plate. After 24 h, a scratch was made in the monolayer of cells using a 200 µL pipet tip. Loose cells were washed away with medium after which the cells were exposed to 3 µM resveratrol and analogues. Photographs of the wound were taken at t=0 and t=48, and the wound area was calculated using ImageJ. The migration assay was repeated 3 times with a replicate of 3 in each experiment.

2.5. AHR CALUX assay

The human HG2L7.5C1 CALUX cell line was created by a stable transfection of an AHR responsive firefly luciferase reporter gene plasmid pGudLuc7.5 and was a kind gift from Prof. M.S. Denison (University of California, Davis, CA). The HG2L7.5C1 cells were cultured in MEM alpha medium (Invitrogen) supplemented with 10% FBS and 1% pen/strep. The cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C, and subcultured twice a week. The cells were seeded at a density of $4*10^4$ cells/well in a 96-well plate. Cells were exposed to ranges of resveratrol and analogues for 24 h with and without 10 µM AHR antagonist CH-223191 (CAS 301326-22-7, Calbiochem), and 0.1% DMSO was used as a negative control for AHR activation. The experiment was finished by lysing and mixing the cells with luciferine reagent consisting 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.7 mM $MgSO_4.7H_2O$, 0.1 mM EDTA, 22.3 mM DTT, 261 µM coenzyme A, 470 µM luciferine and 530 µM ATP (pH 7.8). Luminescence was measured directly after as a proxy for AHR activation. MTT cell viability assays were performed to identify cytotoxicity. The AHR CALUX assay was repeated 3 times with a replicate of 3 in each experiment.

2.6. CYP1A1 activity

CYP1A1 activity in the MCF-7 cells was measured by a CYP1-mediated 7-ethoxyresorufin O-deethylation (EROD) activity assay. The MCF-7 cells were seeded at a density of $3*10^4$ cells/well in a 96-well plate. After 2 days, cells were exposed to ranges of resveratrol and its analogues for 24 h with and without 10 µM AHR antagonist CH-223191 (CAS 301326-22-7, Calbiochem), and 0.1% DMSO was used as a negative control. The experiment was finished by washing the cells and adding DMEM medium supplemented with 50 mM $MgCl_2$, 50 µM 7-ethoxyresorufin, and 10 µM dicoumarol. Fluorescence was measured at an excitation wavelength of 530 nm and emission wavelength of 590 nm. A resorufin calibration curve was used for quantification of the CYP1A1 activity [34]. The EROD assay was repeated 3 times with a replicate of 3 in each experiment.

2.7. Cell cycle

The MCF-7 and MCF-10A cells were seeded with a density of $4*10^5$ cells/well on a 12-well plate. Cells were exposed to 10 µM (MCF-7) and 30 µM (MCF-10A) resveratrol and TMS. 10 µM 2-methoxyestradiol (2-$MeOE_2$) was used as a positive control for a cell cycle arrest in the G2/M phase [35]. After 20 h of incubation, the cells were fixated with 70% ethanol until further analysis. Cell cycle analysis was conducted by staining the cells with propidium-iodide (PI, Sigma, the Netherlands). PI content was measured with the use of flowcytometry. FlowJo was used to analyze the distribution of cells in each phase of the cell cycle, set at <G1, G1, S, and G2/M phase. Multinucleate cells or otherwise aberrant cells were excluded from gates (>G2).

2.8. mRNA isolation and RT-qPCR

The MCF-7 and MCF-10A cells were seeded with a density of $2*10^5$ cells/well on a 12-well plate. Cells were exposed for 48 h to 3 µM (MCF-7) and 10 µM (MCF-10A) resveratrol and TMS. RNA was isolated with the phenol-chloroform extraction method using RNA Instapure (Eurogenetec, Liege, Belgium). Purity and concentration of the isolated RNA were determined were determined spectrophotometrically at an absorbance wavelength of 260 and 280 nm. Complementary DNA (cDNA) was synthesized with 1 µg RNA using the iScript cDNA Synthesis Kit (BioRad), 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 96-well green shell PCR plate (Bio-Rad, Veenendaal, the Netherlands) using the iQ5 cycler (Bio-Rad, Veenendaal, the Netherlands). A PCR mastermix was made containing 12.5 µl of IQ SYBR Green Supermix, 1 µl of a forward and reverse primer (each with a concentration of 10 uM) and 0.5 µl of water per sample. For the qPCR, 10 µl of cDNA of each sample was added to 15µl of the PCR mastermix. Oligonucleotide sequences of the primers were; β-actin, FW TTGTTACAGGAAGTCCCTTGCC, RV ATGCTATCACCTCCCCTGTGTG; CYP1A1, FW CAGAAGATGGTCAAGGAGCA, RV GACATTGGCGTTCTCATCC; CYP1B1, FW CGGCCACTATCACTGACATC, RV CTCGAGTCTGCACATCAGGA; Nqo1, FW GGATTGGACCGAGCTGGAA, RV AATTGCAGTGAAGATGAAGGCAAC; IL-8, FW CTCTTGGCAGCCTTCCTGATT, RV TATGCACTGACATCTAAGTTCTTTAGCA. Primer-specific annealing temperatures were 60 °C for all primers used. Gene expression was determined using the ΔΔCq method of relative quantification where gene expression in the cultures was normalized to β-actin.

2.9. Data analysis

All data is expressed as means + standard deviation of three independent experiments (N) that were performed in triplicate (n). ImageJ was used to analyze particles in the snapshots taken to calculate the migration of the cells. Data was analyzed using GraphPad Prism 7. Student's t-tests were performed to determine statistical difference of the mean between the treatments in the viability, migration, AHR CALUX, CYP1A1 activity, cell cycle and gene expression assays.

3. Results

3.1. Effects of resveratrol and its analogues on tumor cell viability

Human MCF-7 breast cancer cells were exposed to resveratrol and its analogues to assess their effect on tumor cell viability. The cells were exposed for 10 days to a concentration range up to 30 µM for all compounds, in combination with 20 pM E2 to induce proliferation. E2 concentration-dependently stimulated MCF-7 cell proliferation with an EC50 value of 20 pM E2 [36]. Oxyresveratrol and resveratrol did not statistically significantly affect MCF-7 cell viability at concentrations up to 30 µM (Figure 2, upper panel). In contrast, resveratrol analogues pinostilbene, pterostilbene, and tetramethoxystilbene (TMS) statistically significantly reduced MCF-7 tumor cell viability with a 50% reduction of cell viability at 14.2 µM for pinostilbene, 15.6 µM for pterostilbene, and 3.6 µM for TMS (Figure 2, lower panel).

3.2. Effects of resveratrol and its analogues on MCF-7 tumor cell migration

A wound healing assay was used to assess the effects of resveratrol and its analogues on MCF-7 tumor cell migration. After inflicting a scratch in the MCF-7 monolayer, the cells were exposed for 48 h to 3 µM for all compounds or 0.1% vehicle control (DMSO) and the wound surface area was measured as marker for cell migration. After 48 h, the wound surface area in the vehicle-treated control monolayer was approximately 40% of the original wound surface area. Effects on cell migration of resveratrol and its analogues were calculated relative to the migration of the vehicle-treated control cells. No statistically significant difference in cell migration was observed between the control treatment and the resveratrol treatment. Oxyresveratrol reduced MCF-7 tumor cell migration with 12.1 ± 7.2%, pinostilbene with 15.2 ± 3.8% and pterostilbene with 13.2 ± 11.6%. Only TMS statistically significantly reduced MCF-7 tumor cell migration with 37.5 ± 14.8% (Figure 3).

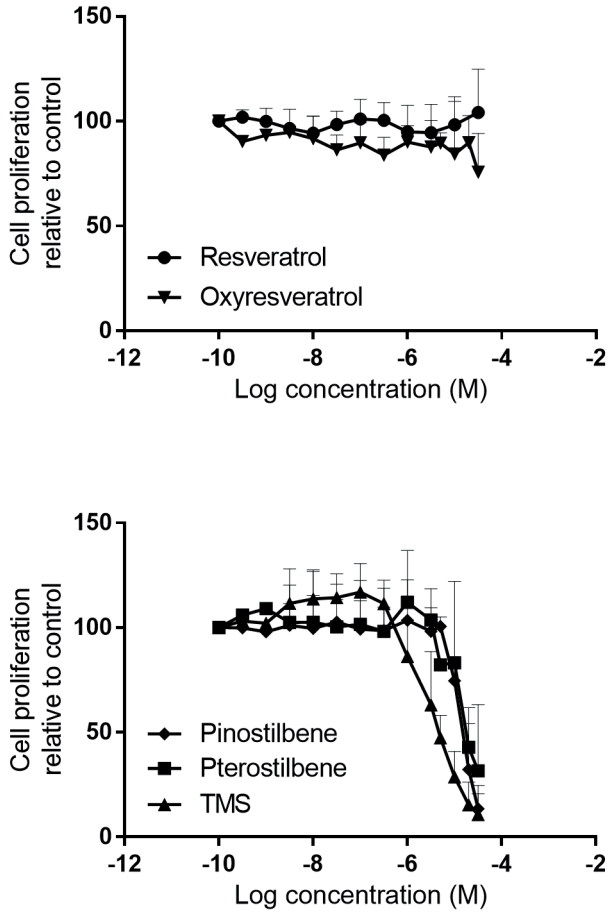

Figure 2. Cell viability assay in MCF-7 cells. Cells were exposed to a concentration range (0-30 µM) of resveratrol and four of its analogues in combination with 20 pM E2. Data is expressed relative to the vehicle treated cells (0.1% DMSO + 20 pM E_2) that was set to 100% and are expressed as mean ± standard deviation (N=3), and adjusted for the background values.

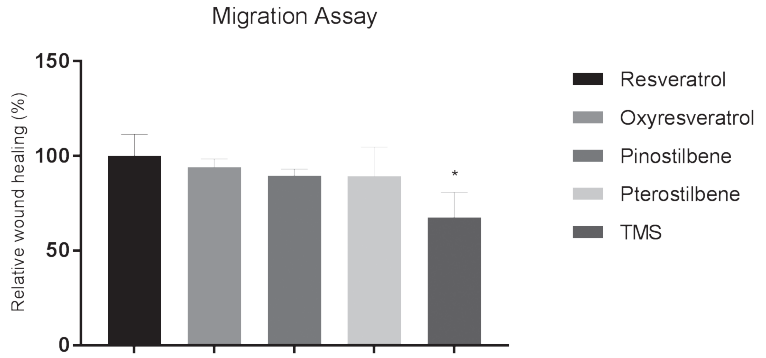

Figure 3. Wound healing assay in MCF-7 cells. Cells were exposed to 3 µM resveratrol and its analogues for 48 h. Wound surface area is measured as marker for MCF-7 tumor cell migration. Data is expressed as mean wound surface area ± standard deviation (N=3), relative to the vehicle-treated control (0.1% DMSO) cells. * Statistically significantly different from vehicle-treated control cells (P≤0.05).

3.3. Effect of resveratrol and its analogues on AHR activation and CYP1A1 activity

To assess the potency of resveratrol and its analogues to activate the AHR, a reporter gene assay was performed. HG2L7.5C1 cells, which are stably transfected human hepatoma HepG2 cells with an AHR responsive firefly luciferase reporter gene plasmid, were treated for 24 h with concentration ranges of the compounds (0-30 µM) with and without AHR antagonist CH-223191 (10 µM). Resveratrol did not cause a statistically significant effect on AHR activity in HG2L7.5C1 cells up to 30 µM (Figure 4, left panel). In contrast, oxyresveratrol, pinostilbene, pterostilbene and TMS statistically significantly activated the AHR. AHR activation was maximal after exposure to 20 µM oxyresveratrol, 5 µM pterostilbene and 3 µM TMS, after which the activity declined (Figure 4, left panels). This was not due to cytotoxicity as measured by an MTT assay (data not shown). No maximum AHR activation could be determined for pinostilbene. The EC50 value for AHR activation was 5 µM for oxyresveratrol, 2.6 µM for pterostilbene and 2.0 µM for TMS. Addition of AHR antagonist CH-223191 negated AHR activation by oxyresveratrol, pterostilbene and TMS, indicating an AHR-mediated effect of these resveratrol analogues. Co-exposure to CH-223191 had no significant effect on AHR activation by pinostilbene at the three highest exposure concentrations.

In addition to AHR activation, the potency of resveratrol and its analogues to induce CYP1A1 activity was assessed by means of an EROD assay. MCF-7 cells were treated for 24 h with concentration ranges of the compounds (0-30 µM) with and without AHR antagonist CH-223191 (10 µM). Pterostilbene and TMS concentration-dependently induced EROD activity in MCF-7 cells with EC50 values of 5 µM and 0.7 µM, respectively (Figure 4, right panels). The induction of EROD activity by pterostilbene and TMS was inhibited by the AHR antagonist CH-223191, again confirming an AHR-mediated effect of these analogues. Resveratrol, oxyresveratrol and pinostilbene did not cause a statistically significant effect on EROD activity in MCF-7 cells after exposure to concentrations up to 30 µM (Figure 4, right panels).

3.4. Effect of resveratrol and TMS on cell cycle status in MCF-7 and MCF-10A cells

Overall, the viability, migration, AHR reporter gene assay and EROD assays revealed that resveratrol analogue TMS displays the strongest anti-tumorigenic properties and is the most potent AHR activator of all the analogues tested. Therefore, TMS was studied in more detail in MCF-7 cells and the non-tumorigenic breast epithelial cell line MCF-10A.

Cell cycle status was determined in MCF-7 and MCF-10A cells after a 20-h exposure to resveratrol and TMS to concentrations known to activate the AHR. 10 µM 2-methoxyestradiol (2-MeOE$_2$) was used as positive control for G2/M cell cycle arrest. Indeed, 2-MeOE2 reduced the cell cycle status of MCF-7 cells in the G1 phase with 30%, and increased the cell cycle status with 21% in the G2/M phase (Figure 5, upper panel). The cell cycle status of the MCF-7 cells was not affected by resveratrol. Cell cycle status of MCF-7 cells shifted from the G1 phase to the G2/M phase upon exposure to 10 µM TMS. The fraction of cells in the G1 phase were statistically significantly reduced with 27% after exposure to TMS, while the fraction of cells in the G2/M phase increased with 18%.

In MCF-10A cells, the number of cells in the G1 phase was reduced with 41% by 2-MeOE2, and increased in the G2/M phase with 33%. In contrast to MCF-7 cells, resveratrol, but not TMS, did affect cell cycle status in MCF-10A cells (Figure 5, lower panel). Exposure to 30 µM resveratrol resulted in a shift in cell cycle status from the G1 to the S phase. The fraction of cells in the G1 phase reduced with 16% after the exposure to resveratrol, while the fraction of cells in the S phase increased with 12%. In contrast to MCF-7 cells, TMS did not

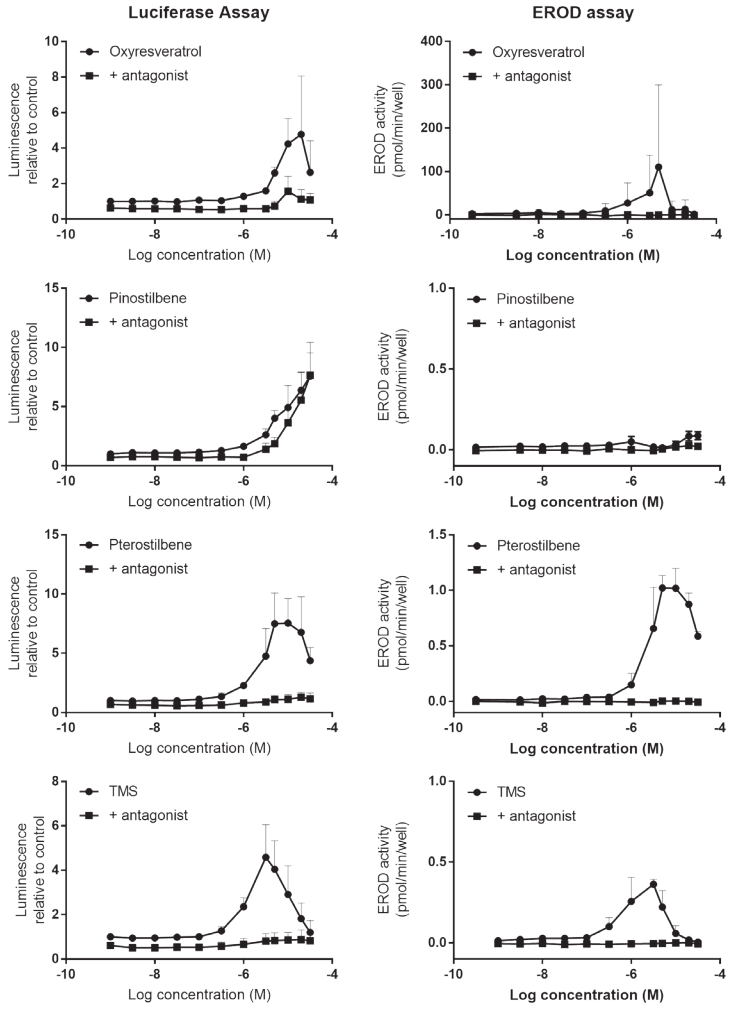

Figure 4. AHR reporter gene assay in HG2L7.5C1 cells (left panels) and EROD activity in MCF-7 cells (right panels). Cells were exposed to resveratrol, oxyresveratrol, pinostilbene, pterostilbene, and tetramethoxystilbene (TMS) with concentration ranges up to 30 µM and in combination with 10 µM CH-223191 AHR antagonist. Data are expressed as means of three independent experiments with standard deviation (N=3). [a] Statistically significantly different from vehicle-treated control cells and from treatment with inhibitor (P≤0.05); [b] Statistically significantly different from vehicle-treated control cells (P≤0.05).

affect cell cycle status in MCF-10A cells, even when the cells were exposed to a higher concentration of 30 µM TMS (Figure 5, lower panel).

3.5. Effect of resveratrol and TMS on gene expression

Gene expression patterns were analyzed in MCF-7 and MCF-10A cells after exposure to resveratrol or TMS to further elucidate the mechanism by which these compounds exert their effects.

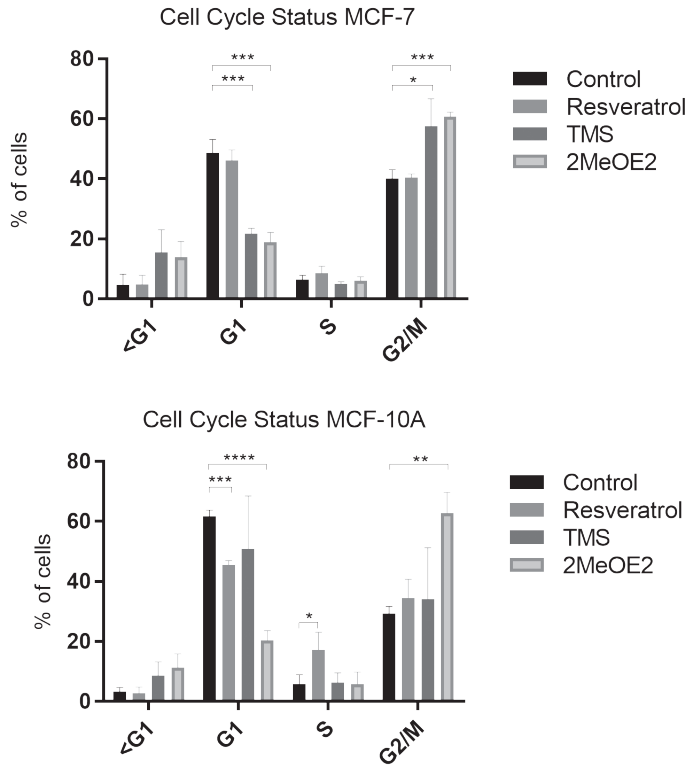

Figure 5. Analysis of the cell cycle status of MCF-7 and MCF-10A cells. Cells were exposed to 10 µM (MCF-7) or 30 µM (MCF-10A) resveratrol or TMS, 0.1% DMSO (vehicle control) and 10 µM 2-MeOE2 (positive control) for 20 h. The bars represent the fraction of the cells distributed over each phase of the cell cycle and are expressed as mean ± standard deviation (N=3). Asterisks indicate statistically significant differences from vehicle-treated control cells with * P≤0.05, ** P≤0.01, *** P≤0.001, and **** P≤0.0001.

In MCF-7 cells, CYP1A1 gene expression increased 289-fold and CYP1B1 expression increased 4.9-fold after a 48-h exposure to 3 µM TMS. CYP1A1 or CYP1B1 gene expression did not significantly change after exposure to 3 µM resveratrol. Nqo1 gene expression increased 2-fold in MCF-7 cells after a 48-h exposure to 3 µM TMS, while there was no change in Nqo1 expression

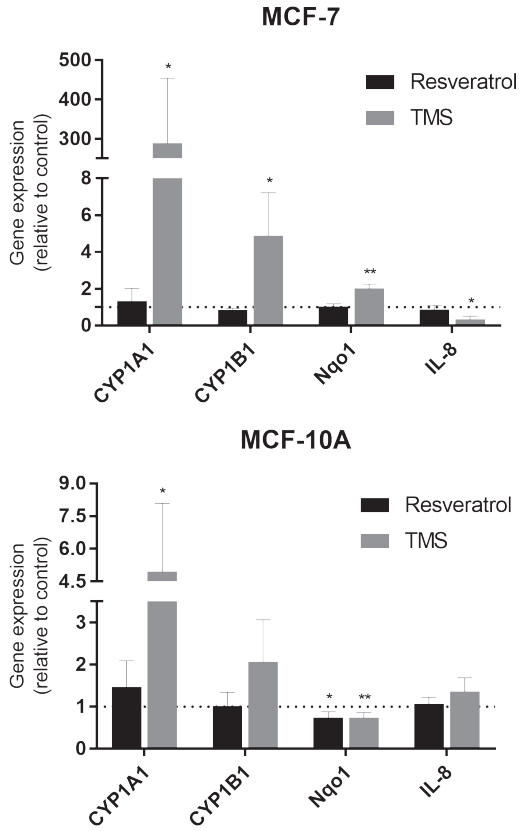

Figure 6. Changes in gene expression of AHR, CYP1A1, CYP1B1, Nqo1 and IL-8 after a 48-h exposure in MCF-7 (upper panel) and MCF-10A cells (lower panel). Cells were exposed to resveratrol or TMS (3 µM in MCF-7 and 10 µM in MCF-10A). Data is expressed relative to DMSO treated controls that were set to 1 (represented by the dashed line) and expressed as mean and ± standard deviation with N=3 for MCF-7 and N=4 for MCF-10A. * Statistically significantly different from control treated cells (P≤0.05) and ** significantly different from control (P≤0.01).

after exposure to 3 μM resveratrol. In addition, IL-8 gene expression decreased 3-fold in MCF-7 cells when exposed to 3 μM TMS, while no significant change in IL-8 gene expression was observed upon exposure to 3 μM resveratrol. AHR gene expression did not statistically significantly change in MCF-7 cells upon exposure to either 3 μM TMS or resveratrol (Figure 6).

In the non-tumorigenic MCF-10A cells, CYP1A1 gene expression also increased, albeit only 5-fold, after a 48-h exposure to 10 μM TMS. CYP1B1 gene expression increased 2-fold after exposure to 10 μM TMS, yet this increase was not statistically significant. CYP1A1 and CYP1B1 gene expression in MCF-10A cells did not change upon exposure to 10 μM resveratrol. In contrast to MCF-7 cells, Nqo1 gene expression decreased 1.4-fold upon exposure to 10 μM resveratrol or TMS in MCF-10A cells. IL-8 gene expression increased 1.4-fold when MCF-10A cells were exposed to 10 μM TMS, although this increase was not statistically significant. Resveratrol did not affect IL-8 gene expression in MCF-10A cells. Similar to MCF-7 cells, AHR gene expression did not statistically significantly change by resveratrol or TMS exposure in MCF-10A cells (Figure 6).

4. Discussion

In this study, the anti-tumor properties of resveratrol and several structural analogues on MCF-7 tumor cell growth and migration were assessed. The potency of these compounds to initiate anti-tumorigenic effects seemingly corresponds with their potency to activate the AHR. TMS was the most potent anti-tumorigenic resveratrol analogue. Noticeably, TMS caused a cell cycle arrest in the malignant MCF-7 cells, but not in non-tumorigenic MCF-10A breast epithelial cells. Although TMS activates the AHR in both MCF-7 and MCF-10A cells, this appears to result in cell-type specific actions possibly related to tumorigenic status.

4.1. Anti-tumor properties of resveratrol and its analogues

Resveratrol and its analogues have previously been described to possess anti-tumor properties, such as inhibition of tumor cell viability, proliferation and migration. In present study, resveratrol did not affect tumor cell growth, not even at the highest exposure concentration of 30 μM. Earlier reports mention inhibitory effects by resveratrol on MCF-7 cell proliferation, yet higher exposure concentrations were used (up to 300 μM) [37, 38]. The anti-tumor properties of the compounds tested appear to increase with the number of methoxy substitutions to the stilbene backbone of resveratrol. An additional hydroxy group in the stilbene backbone of resveratrol, like in oxyresveratrol, did not significantly affect tumor cell viability nor migration in our study. However, if the hydroxy group was replaced by a methoxy group on the stilbene backbone, an increase in anti-tumor properties, as measured by a viability and migration assay, was observed. Pinostilbene, pterostilbene and TMS with respectively one, two and four methoxy groups, all reduced tumor cell viability and migration in MCF-7 cells with TMS being the most potent compound. Others have also reported an inhibition of cell proliferation by methoxylated resveratrol derivatives, *e.g.* pterostilbene in MCF-7 cells [39], trimethoxyl stilbene in a A549 lung cancer cells [40] and tetramethxoystilbene in MCF-7 cells [41]. The observed concentration at which TMS inhibited MCF-7 cell migration (3 μM) is in the same order of magnitude as the 50% decrease of MCF-7 cell viability (3.6 μM). In our experimental set-up, the 50% decrease in viability assay was obtained after 8 days of exposure to TMS, hence involving multiple cell cycles and cell divisions. The average doubling time of MCF-7 cells is 30-40 h in regular culture medium. Therefore, after a period of only 48 h, the effect of TMS on cell proliferation in the migration assay in present study was considered to be relatively minor.

Analysis of the cell cycle distribution showed that resveratrol did not affect the cell cycle progression of malignant MCF-7 cells at a concentration of 10 μM, while the non-tumorigenic MCF-10A cells accumulated in the S-phase of the cell cycle at 30 μM resveratrol. This cell phase specific accumulation for resveratrol has been reported previously for MCF-7 and MCF-10A-Tr cells, at slightly higher or comparable concentrations as in our experiments [42, 43]. Joe *et al*. (2002) also reported an S-phase arrest by resveratrol in MCF-7 cells at 300 μM as well as in the HCE7 human esophageal squamous carcinoma and HL60 human promyelocytic leukemia cell lines [38]. The concentration resveratrol in present study was an order of magnitude lower than the concentration used by Joe *et al*. (2002), which may explain the absence of an S-phase arrest by resveratrol in the MCF-7 cells. TMS induced a shift from the G1 phase to the G2/M phase causing a cell cycle arrest in the MCF-7 cells, which was not observed for resveratrol. In line with our findings, other resveratrol analogues with four methoxy-groups (3,4,4',5-tetramethoxy-*trans*-stilbene) induced the same G2/M phase cycle arrest in a prostate cancer cell line [44], ovarian cancer cell line [45] and in MCF-7 and HepG2 cell lines at similar concentrations as

our experiments [41]. In addition, we noticed a slight, yet not statistically significant, increase in cells in the sub-G1 phase upon TMS exposure. This suggests an increase in apoptotic cells, which corroborates with the observed decrease in cell viability by TMS in our study.

Sangjun *et al*. (2009) studied the effects of several methoxy additives to *cis*-stilbenes derivatives in MCF-7 cells and found that methoxy groups at the *para* position of the left aromatic ring to be a key position to induce cell cycle arrest [35]. There was indeed a methoxy group positioned at that site of 3,4,4′,5-TMS used by Horvath *et al*. (2007), Piotrowska *et al.* (2012) and Androutsopoulos *et al*. (2011) who observed a cell cycle arrest by TMS. However, while 2,3′,4,5′-TMS in our study did induce a cell cycle arrest, it did not hold a methoxy group at this particular site. Possibly, that specific methoxy position may be of more importance in the *cis*-stilbenes than for the *trans*-stilbenes, as was used in this study.

Strikingly, TMS selectively induced a cell cycle arrest in the malignant MCF-7 cells and not in the non-tumorigenic MCF-10A cells, while resveratrol only induced an arrest in the MCF-10A cells and the positive control 2-MeOE2 induced a cell cycle arrest both cell lines. The endogenous estrogen metabolite 2-MeOE2 has been shown to cause a cell cycle arrest and induce apoptosis via inhibition of kinase pathways and Bcl2 downregulation [46]. To the best of our knowledge, no other studies have described the effects of TMS on the cell cycle of MCF-10A cells. The differences in anti-migration and cell cycle arrest properties of TMS in the two cell lines may be explained by differences in AHR activation. Cell type specific differences may arise from different AHR ligand binding pockets, different recruitment of and/or cell-type specific expression of co-activators/repressors and different availability of ligand DNA recognition sites between the cell lines [47]. The involvement of differential AHR activation between the cell lines is supported by clear different gene expression patterns in MCF-7 and MCF-10A cells that was inflicted by TMS exposure (further discussed below).

4.2. AHR activation by resveratrol and its analogues

Several studies have previously described that resveratrol can activate the AHR and induce CYP1A1 activity [5, 48]. In our study using the AHR reporter cell line HG2L7.5C1, resveratrol did not activate the AHR at concentrations up to 30 μM. However, adding methoxy groups to the stilbene backbone significantly increased the potency of the resveratrol analogues to activate the AHR with TMS being the most potent analogue followed by pterostilbene and oxyresveratrol. The increased AHR activity was corroborated with an increase in CYP1A1/1B1-mediated EROD activity in MCF-7 cells. Interestingly, while pinostilbene did induce AHR activity in the reporter gene assay, it could not be negated by adding the AHR antagonist CH-223191. In addition, pinostilbene did not induce EROD activity in MCF-7 cells. Possibly, pinostilbene binds to a different activation site of the AHR than the antagonist CH-223191 and the other resveratrol analogues tested. Another interesting finding was the fact that all resveratrol analogues caused a decrease in AHR activation as well as EROD activity at higher concentrations. It has been reported that resveratrol can inhibit CYP1A1 and CYP1B1 activity in human recombinant supersomes with a reported IC50 of 26 μM and 11 μM, respectively [49]. TMS exhibited stronger CYP1A1 and CYP1B1 inhibitory properties with previously reported IC50 values of 300 nM and 6 nM respectively, which also indicates that TMS exhibits a selective catalytic inhibition of CYP1B1 activity [50]. This may explain the decrease in EROD activity at higher concentrations of the resveratrol analogues that we observed. In present study, CYP1A1 and CYP1B1 gene expression was induced by TMS in both MCF-7 and MCF-10A cell lines. CYP1A1 and CYP1B1 gene expression was more strongly induced in the MCF-7 cells compared to MCF-10A cells, which may relate to observations that the AHR is overexpressed in many tumor cells [17, 51]. Piotrowska *et al*. (2012) studied the effect of 3,4,4′,5-tetramethoxy stilbene in two ovarian cancer cell lines. Although they also observed an inhibition of proliferation and induction of cell cycle arrest by TMS, they found that TMS decreased CYP1A1 and CYP1B1 mRNA and protein expression after 24 h of exposure [45]. Possibly, this inhibitory effect is attributed to the different structural arrangement of the TMS that was used in that study. Additionally, the formation of a TMS metabolite is suggested to be responsible for the modulation of CYP1A1 and CYP1B1 expression [52]. Similar to our study, Einem Lindeman *et al.* (2011) also used 2,3′,4,5′-tetramethoxystilbene and did not observe an inhibitory effect on CYP1A1 and CYP1B1 expression in MCF-7 cells [53].

While AHR activation is often associated with dioxin-like toxicity, such as chloracne in humans, it should be recognized that the activation of the AHR and concurrent induction of CYP1A1 and CYP1B1 by itself do not necessarily indicate toxicity. Nowadays, the induction of CYP450 isoforms is usually considered as the earliest and most sensitive signal of AHR activation. A wide

range of non-dioxin-like compounds, such as polycyclic aromatic hydrocarbons (PAHs) but also other natural compounds like indole-3-carbinol, are causing CYP1A1 and CYP1B1 induction without expressing the classical toxic endpoints associated with halogenated dioxins, dibenzofurans or PCBs. Investigating the effects on CYP1A1/1B1 gene expression and activity of potential anti-cancer agents are especially important with respect to the role of CYPs in formation of genotoxic estrogen metabolites. CYP1A1-mediated estrogen metabolism yields predominantly 2-hydroxylated estrogens, which are considered to protect against tumor formation. In contrast, CYP1B1 catalyzes estrogen 4-hydroxylation, which may lead to quinones that react with DNA and as a result may initiate cancer [8]. Further studies should be performed to elucidate the effect of TMS on estrogen metabolism.

While gene expression of CYP1A1 and CYP1B1 was increased in both MCF-7 and MCF-10A cells, gene expression of Nqo1, a hallmark for Nrf2 pathway activation, was increased by TMS in MCF-7 cells but decreased in the MCF-10A cells. This indicates that TMS activates a pathway that is induced in response to cellular oxidative stress, but only in the malignant breast tumor cells. Resveratrol has also been reported to induce Nqo1 expression in the human K562 leukemia cell line [54]. However, concentrations used in that study were 10-fold higher than in our experiments, which may explain the observed absence of Nqo1 induction by resveratrol in our study. Interesting is the finding of Lu *et al*. (2008) [55] who describe a slight increase in Nqo1 protein expression and enzymatic capacity 48 h after exposure to resveratrol (50 μM) in MCF-10F cells, which are similar to MCF-10A regarding for example hormone receptor status [56]. Our results show a decrease in Nqo1 gene expression after 48 h in MCF-10A. This does not exclude that resveratrol and TMS do not induce an oxidative stress response in these cells, as gene expression changes often do not corroborate with protein expression changes when determined at the same time point. An increase in Nqo1 capacity may protect the cell against formation of quinones and potential genotoxic events by estrogen metabolites.

Gene expression of IL-8 was reduced by TMS in MCF-7, but no clear effect on IL-8 gene expression was observed in the MCF-10A cells. Others have associated AHR activation with IL-8 downregulation which can be linked to cell cycle arrest via PTEN/Akt signaling pathways [12, 57, 58]. These studies are in line with our findings that TMS exposure reduces IL-8 gene expression and induces a cell cycle arrest in MCF-7 cells. The lack of IL-8 downregulation in MCF-10A cells may also explain the absence of a cell cycle arrest by TMS in that cell line.

5. Conclusions

Taken together, our data suggest that the methoxylated analogues of resveratrol hold more potent anti-tumor properties than resveratrol itself, which may be mediated by the AHR. TMS, but not resveratrol, selectively inhibited the cell cycle of the malignant MCF-7 cells, but not the non-tumorigenic MCF-10A cells. The methoxylated analogues of resveratrol could therefore be potentially more useful as anti-cancer agents than resveratrol itself. The findings from our study and others suggest that the anti-cancer properties of TMS may be dual: it may act as anti-tumor agent through inhibition of cell viability, migration and cell cycle arrest in tumor cells, but also as cancer chemo-preventive agent in non-malignant breast cells via inhibition of CYP1B1-mediated estrogen metabolism and possibly via Nqo1 and IL-8-mediated pathways.

6. Conflict of interest

None of the authors have competing interests to declare. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Chapter 7

General discussion

1. Summary

Rationale

Endometrial diseases affect up to approximately 10% of women in their reproductive age. Yet, current testing strategies in chemical risk assessment do not adequately address endometrial health, partly because good (animal-free and human relevant) models are lacking. The overall aim of this thesis was to address the species-specific differences between human and rat endometrium, as well as to assess tissue-specific and ligand-specific differences in response to aryl hydrocarbon receptor (AHR) activation.

Summary of experimental results

An *ex vivo* 3D endometrial model was developed in **Chapter 2** using healthy human primary endometrial cells. This endometrial model consists of two layers; a bottom layer where an extracellular matrix scaffold encloses endometrial primary stromal cells and an upper layer of endometrial primary epithelial cells to allow polarization of the cells. The endometrial model was subjected to hormonal stimuli to mimic the proliferative phase (estrogen-dominant) and secretory phase (progesterone-dominant) of the menstrual cycle. In addition, we assessed the effects on endometrial responses upon AHR ligands activation. A comparable model was established using primary rat endometrial cells, to assess species- specific differences in endometrial responses. This chapter showed that steroid hormones modulated the gene expression of steroid receptors and other marker genes comparable to the human *in vivo* situation. Also, significant and opposing differences were observed between responses in the human and rat endometrial models with respect to steroid hormones, as well as the AHR ligands TCDD, laquinimod and its primary metabolite DELAQ.

In **Chapter 3** the interplay between the AHR and estrogen signaling in endometrial tissue was further explored in primary endometrial epithelial cells. Again, we observed the opposing effects of AHR ligands between the human and rat endometrial cells. Differential regulation of this receptor between human and rat tissue was also observed with respect to hormonal action. In addition, the inhibitory effect on estrogen signaling upon AHR activation was only observed in the human endometrial cells, and not in the rat endometrial cells.

The research in Chapter 2 and Chapter 3 showed that the rat endometrium does not reflect the human situation. We assessed another aspect of human-rat differences within the hormonal regulatory feedback system in **Chapter 4**. The effect of AHR activation on prolactin secretion from the pituitary in rats was assessed. Prolactin secretion can stimulate the production of progesterone in rat ovaries. Therefore, aberrant prolactin secretion by AHR ligands can also evoke effects on the endometrium in rats. In Chapter 4, the prolactin regulation was compared between rat primary pituitary cells and two rat pituitary cell lines, GH-3 and RC-4BC. It was observed that these pituitary cell lines do not reflect prolactin regulation *in vivo*, as they showed different responses to prolactin inhibitor quinpirole and prolactin stimulant thyrotropin-releasing hormone compared to the primary pituitary cells. In addition, it was demonstrated that AHR ligands TCDD and DELAQ, although both activating the AHR, exerted different effects on prolactin gene expression in primary pituitary cells.

The activation of AHR ligands TCDD, DELAQ, resveratrol and its analogues were also assessed in endometrial adenocarcinoma (**Chapter 5**) and breast cancer (**Chapter 6**) cell lines, to address tissue specific differences of AHR activation. In Chapter 5, we observed different effects of resveratrol and its analogues between two endometrial cell lines Ishikawa and ECC-1. It appeared that the resveratrol analogue tetramethoxystilbene (TMS) exerted stronger anti-tumorigenic properties in ECC-1 cells, whereas it did not affect Ishikawa cells. In Chapter 6, we observed anti-tumorigenic properties of the resveratrol analogues in the MCF-7 breast cancer cell line. These effects were stronger with increasing methoxy-substitutions on the stilbene backbone of these analogues. Moreover, the most potent resveratrol analogue TMS inhibited the cell cycle of MCF-7 cells, but did not affect the cell cycle of the non-tumorigenic breast epithelial cell line MCF-10A.

2. General discussion

While the prevalence of uterine diseases has been linked to the exposure of environmental contaminants and AHR activation, it is very challenging to study the exact modes of action underlying these diseases. Even though the mechanisms behind the etiology of *e.g.* uterine diseases between humans and rodents are largely similar for uterine tumors, there are also mechanisms in rats

which are not of relevance to humans, especially the progesterone stimulating effect of prolactin [1].

Several studies have indicated a relation between endometrial diseases and exposure to AHR ligands [2, 3]. Although the AHR is evolutionary strongly conserved, there are significant differences in effects between species, tissues and ligands upon activation of this receptor. For example, the lethal potency of TCDD varies over a 1000-fold among different animal species upon oral administration [4]. The research in this thesis focused on developing a human-relevant endometrial model in which we assessed the endometrial responses upon activation of the AHR. This allowed us to provide a better understanding of species differences in endometrial responses, specifically on hormonal responses and the activation of the AHR thereon.

3. Species-specific differences in endometrium

Hormonal responses and AHR activation

A distinct difference between the primary human and the rat endometrial models is the effect of hormones on the expression of the AHR. Hormones, especially progesterone, lower the expression of the AHR in the rat endometrial cells, whereas hormones did not affect this expression in the human cells (Chapter 2 and 3). This reduced AHR gene expression coincided with a reduced induction of CYP1A1 and CYP1B1 gene expressions when the AHR was activated. Again, this was not observed in the human cells after exposure to hormones. This reduced AHR gene expression is in line with observations in Wistar rats after treatment with hormones *in vivo* [5]. It is however unclear what this reduced AHR expression in the endometrium would imply for the translation of *in vivo* studies. Especially since it has been reported that while AHR gene expression decreased upon hormonal exposure, uterine AHR protein levels increased [5].

Although a cross-talk between the estrogen receptor (ER) and the AHR is identified in humans and experimental species, a direct cross-talk between progesterone (receptor) and the AHR unique to rats has not extensively been described. In addition, progesterone may also indirectly affect AHR-ER cross-talk, as progesterone has an inhibitory role to estrogen signaling in the uterus in both species [6]. AHR activation has often been reported to reduce estrogen signaling as a result of the AHR-ER cross-talk, as well as *vice versa*. This cross-talk can be achieved by interference with ER-DNA binding to specific estrogen responsive elements than contain overlapping dioxin response element sequences. This may inhibit the expression of estrogen-target genes, stimulate ER proteasomal degradation, increase CYP-mediated estrogen metabolism or other as of yet unidentified mechanisms [4, 7–9]. Exposure to TCDD has been shown to reduce estrogen-dependent tumors in rats and reduce the estrogen-dependent proliferation of human endometrial cell lines [10–13]. These findings are not in line with the lack of anti-estrogenicity that was observed in our studies with rat endometrial epithelial cells treated with TCDD and DELAQ (Chapter 3). It is possible that higher concentrations of AHR ligands would have evoked an anti-estrogenic response via a stronger activation of the AHR. Although, maximal CYP expression was already approximately 300-fold compared to the control cells, which indicates already a high level of AHR activation after treatment with both TCDD and DELAQ. Kuo et al. 2013 showed that in a human lung adenocarcinoma cell line the anti-estrogenicity of AHR ligands depends on the balance between the expression of both ER and AHR [14]. A relatively high expression of the AHR even diminished the anti-estrogenic effects of TCDD. This may explain the lack of anti-estrogenic effects of AHR ligands as observed in the rat endometrial tissue, even though the AHR activation level was high.

In human endometrial cells we did observe an anti-estrogenic effect as measured by the estrogen-dependent gene expression of GREB1 by DELAQ. This effect may also have been present with higher concentrations of TCDD that would match the AHR activating potential of DELAQ. The AHR-ER cross-talk is apparently a two-way interaction, because estrogen has also been reported to reduce the expression of certain CYPs in the human endometrium [15]. Such attenuation of CYP gene expression by estrogen exposure has been reported by Willing et al. using immortalized human endometrial cells and in several endometrial cell lines [16, 17]. However, this attenuation was not observed in our primary endometrial cells (Chapter 2 and 3), which indicates that while AHR activation reduced estrogen signaling, estradiol did not reduce AHR signaling in the primary human endometrium.

Yoshida et al. 2004 also did not observe any anti-estrogenic effects in a uterotrophic assay in ovariectomized rats after treatment with the AHR ligand indole-3-carbinol (I3C) [18]. Yet, the incidence of uterine carcinomas increased in a 2-stage uterine model in rats after treatment with I3C *in vivo* [18]. Although

I3C also did not have direct estrogenic effects on the uterus that may have stimulated estrogen-dependent tumor formation, the authors proposed that the I3C-mediated elevation of CYPs in the liver resulted in an increased CYP1B1-mediated estrogen metabolism. The subsequent formation of genotoxic estrogen metabolites could have played a significant role in the effects of I3C on endometrial adenocarcinoma development [19]. Considering the reported anti-estrogenic and thereby "anti-carcinogenic" properties of AHR activation by TCDD, the effects of I3C are seemingly conflicting. It appears that AHR activation not necessarily induces anti-carcinogenic properties in the uterus, but may depend on background levels of estrogens and the balance between anti-estrogenicity and the potential formation of "good" versus genotoxic estrogen metabolites mediated by CYPs that are induced by AHR ligands.

This mechanism is also supported by a study of Hirata et al. 2008, that showed an increased risk of endometrial tumor formation after a reduced CYP1A1 activity [20]. As estrogen metabolism mediated by CYP1B1 can result in depurinating adduct formation that induces DNA damage, a lower CYP1A1/CYP1B1 ratio is a risk factor for endometrial tumor formation [21]. In Chapter 3 of this thesis we also investigated the balance between the induction of CYP1A1 and CYP1B1. We found that the CYP1A1/CYP1B1 ratio was higher in the human endometrium (approximately 1) as compared to the rat endometrium (approximately 0.5 and 0.7) after exposure to the AHR ligands TCDD and DELAQ. This can cause a difference in estrogen metabolism, favoring CYP1B1-mediated E2 metabolism more in the rat compared to human endometrial cells. This can subsequently result in an increased susceptibility to the "tumorigenic" effects of AHR activation in rats. Thus, differences in the CYP1A1/CYP1B1 ratio may be linked to the seemingly contradictory results of "anti-carcinogenic" and "tumorigenic" effects of AHR ligands in rats by different AHR ligands. However, it appears that CYP1B1 hydroxylation differs in rats, as it favors 2-hydroxylation instead of 4-hydroxylation as in humans which can also cause a difference in estrogen metabolism between human and rat endometrial cells [22]. It should also be noted that in Chapter 3, we did not assess the concentration of estrogen metabolites that were formed by CYPs. In future studies it would be informative to assess the 2- hydroxylation/4-hydroxylation ratio of the estrogen metabolites in rat and human endometrial cells and relate this to the CYP gene expression.

In summary, an attenuating effect of progesterone on AHR expression in the rat endometrial cells was observed, whereas this was not observed in the human endometrial cells. In addition, no anti-estrogenic effects of AHR activation were observed in rat endometrial cells, whereas this was observed in human cells. In human endometrium tissue, it appears that AHR activation opposes the effects of estrogen, which may possibly result in a lower endometrial tumor risk. With the use of the endometrium model as described in this thesis, the possible anti-estrogenic effects of AHR ligands can be assessed in more detail. However, the AHR-ER cross-talk is dependent on the cell type and receptor expression patterns, and a constitutively active AHR can also increase tumor risk [14].

AHR ligand-specific differences

In addition to the differences in hormonal responses between the human and rat endometrial models, differences in relative potencies of AHR ligands were also observed between these models (Chapter 2 and 3). Interestingly, the relative degree of AHR activation in the human model, as measured by CYP1A1 expression, was higher by DELAQ compared to TCDD. This was reversed in the rat model, where TCDD more strongly activated AHR mediated CYP activities compared to DELAQ.

In the endometrial co-culture models, the relative response of DELAQ compared to TCDD was approximately a factor 10 *higher* in the human cells, whereas this was a factor 3 *lower* in the rat cells. These differences were even greater in the endometrial mono-culture. Here, DELAQ was approximately a factor 33 stronger than TCDD in the human model and approximately a factor 20 weaker in the rat model. These findings suggest that mono-cultures of primary epithelial cells, do not accurately reflect the *in vivo* situation, possibly due to the lack of interaction of epithelial with stromal endometrial cells.

These species-specific differences of different AHR ligands can be explained by *e.g.* differences in ligand binding pockets on the receptor, differential recruitment and/or availability of co-activators or repressors and differences in available ligand DNA recognition sites between species [23]. These differences can also explain the different types of effects that are evoked by different AHR ligands. For example, TCDD is known for its induction of chloracne in humans, monkeys and rabbits, but this is not observed in other species [4, 24].

The promiscuity of AHR ligand binding pockets also lead to different effects of AHR antagonists. The AHR antagonist CH223191 inhibits the TCDD-induced AHR activation and subsequent gene expression, but may have little effect on other non dioxin-like AHR ligands such as DELAQ, as we have shown in Chapter 4 [25].

At present, it is unclear how the binding pocket of the AHR accommodates so many structurally, different ligands and how the subsequent seemingly differential AHR activation can induce such a diverse array of effects [8]. However, this may explain the differences reported with regard to the "anti-carcinogenic" (i.e. anti-estrogenic) versus "tumorigenic" effects of AHR activation in the rat uterus (as described above). For example reduced incidences of uterine tumors were reported after treatment with TCDD in the rat, but conversely increased incidences of uterine tumors were reported after treatment with I3C. In Chapter 3 of this thesis, we described the (small) difference in CYP1A1/CYP1B1 ratio between these two AHR ligands in rat endometrial cells, whereas no such difference was observed in human cells.

In summary, different AHR ligands can show a reversed relative degree of AHR activation between the human and rat endometrium. This observation gives an additional uncertainty when translating endometrial data from *in vivo* studies to the human situation. For example, when no AHR-mediated effects are observed in the rat endometrium, this may not exclude an effect in the human endometrium. Therefore, the use of human-relevant endometrial models, such as described in Chapter 2, are encouraged when assessing the effects of different AHR ligands in the endometrium.

Tissue-specific differences in AHR response

We not only assessed the endometrial responses to AHR activation between species, but also between tissues. In rats, we therefore compared AHR activation between primary endometrial cells and primary pituitary cells, as well as between primary pituitary and pituitary cell lines.

Ligand specific differences in potency between the AHR ligands TCDD and DELAQ as described earlier were also observed between these different primary tissues from the rat. Where TCDD was approximately 20-fold more potent in AHR activation as measured by CYP1A1 induction in rat endometrial epithelial cells, this induction was 25-fold in primary pituitary cells. A smaller difference in potency was observed between primary pituitary cells and the pituitary cell line GH3. However, in GH3 cells, TCDD was only 1.7-fold more potent in the induction of CYP1A1 when compared to DELAQ, as opposed to the 25-fold induction in primary pituitary cells. These differences in potencies between rat primary tissue and tumor cell lines have not been described earlier. Possibly, this difference is a result of a higher AHR expression in the pituitary tumor cell line, as is often observed in tumor cells [26, 27]. This higher AHR expression may result in a lower maximal induction of *e.g*. CYP1A1, as a result of a saturation level that is achieved earlier [14]. This may explain the difference in efficacy between the two AHR ligands that we studied in both pituitary tumor cell lines.

Another interesting difference between the rat primary endometrial and pituitary cells is the difference in maximal induction of CYP1A1 at similar concentrations of AHR ligands. After treatment with 1 nM TCDD, the maximal induction in endometrial tissue ranged between 950-fold and 1500-fold depending on the type of hormonal treatment, while this induction was only 80-fold in pituitary tissue. This may also be a consequence of differences in basal AHR expression. However, in our studies no absolute AHR gene expression levels were analyzed in the different tissues. Our observations are in line with those of Horn et al. 2002, who showed that significant differences in CYP1A1 expression between rat liver and mammary gland exist [28]. It also appears that, in rats, the expression of CYPs in the mammary gland very much depend on age and hormonal status [29], which may also hold true for endometrial tissue. These tissue specific differences may very well have implications for estrogen metabolism and subsequent activation of pro-carcinogenic metabolites.

In human cells, the response of healthy primary endometrial cells to AHR activation can be compared to human endometrial and liver cell lines. In addition, AHR activation by *e.g.* resveratrol and analogues can be compared between endometrial and breast cancer cell lines.

In primary epithelial endometrial cells, DELAQ was a stronger AHR activator compared to TCDD (Chapter 3) as measured by CYP1A1 gene expression. However, when compared to the human endometrial cancer cell lines, TCDD was a stronger AHR activator compared to DELAQ in both ECC-1 cells and Ishikawa endometrial cells. It is known that tumorigenic tissue often expresses higher levels of the AHR compared to healthy cells [26, 27, 30]. Nevertheless, it

does not explain the reversed potency of the AHR ligands in the healthy tissue *versus* carcinoma cell lines of the endometrium. Moreover, a luciferase reporter gene assay with the human HepG2 hepatoma cell line showed a similar AHR activation profile as these endometrial cell lines. As this HepG2 cell line has been immortalized it would be informative to compare the AHR activating potencies of TCDD and DELAQ in primary hepatocytes with primary endometrial cells.

Interestingly, the maximal CYP1A1 induction also differed between the two primary endometrial models. The average induction by AHR ligands was higher in the endometrial co-cultures, compared to the mono-cultured cells. This suggests that CYP induction in the endometrial stromal cells may be higher compared to endometrial stromal cells, or that the epithelial cells contribute to the CYP expression in stromal cells. This again underlines the differences that are observed between cell types when it comes to AHR activation.

Tissue differences between endometrial and breast cancer cell lines were observed as well. Where the AHR ligand TMS clearly exhibited anti-tumor properties (Chapter 5) by inhibiting cell proliferation, migration and cell cycle in MCF-7 breast cancer cells, no such effect was observed in the Ishikawa endometrial cell line and only on cell proliferation and migration in the ECC-1 cell line. A distinct difference in maximal CYP1A1 induction was also observed between these cell lines. Where TMS induced CYP1A1 expression approximately 300-fold in MCF-7 cells, it only induced this approximately 5-fold in ECC-1 cells, and no induction was observed in the Ishikawa cells. After exposure to other AHR ligands, such as resveratrol and TCDD, the maximal induction was approximately 15-fold higher in the ECC-1 cells compared to the Ishikawa cells (Chapter 4).

In summary, these results indicate distinct differences in AHR-mediated responses between healthy primary tissues and tumorigenic cell lines, but also between endometrium and breast cell lines. Although all these cells are responsive to E2, these act clearly different in response to hormones and/or AHR ligands. In future studies it is important to recognize such differences, especially because in uterine tissue, P4 exerts opposing effects on estrogen. Conversely, P4 stimulates the effects of estrogen in breast (cancer) cells. Most AHR-steroid receptor interactions have been studied in human breast and endometrial carcinoma cell lines [31]. It is questionable if the observed interactions are indeed applicable for real life situations, as our studies showed

that AHR ligands behave differently between tissues, as well as between healthy and tumor cells. It is important to recognize this discrepancy *e.g.* for newly developed pharmaceuticals that can interact with the AHR.

Inter-individual differences

It is known that the expression of CYP enzymes varies considerably between humans, age and gender. For example, catalytic activity of CYP1A1 in human lymphocytes differed 13-fold between individuals and AHR binding affinity differed over 10-fold in human placental cells upon exposure to TCDD [33, 34]. The same inter-individual difference was observed in CYP1A1 gene expression in our human primary endometrial cells with approximately 10 to 15-fold differences in the co-culture as well as the mono-cultures (Chapters 2 and 3).

Besides genetic predisposition, this variability of CYP expression could originate at least in part due to life-style factors such as smoking and diet [35, 36]. This information was not available from our donors due to ethical restrictions. As a result we cannot make any conclusions regarding the cause(s) of our observed high variability between the donors. In addition, genetic factors such as AHR and CYP1A1 and CYP1B1 polymorphisms have been identified in humans, which can also influence the degree of individual CYP inductions in response to AHR ligands [37]. These differences can also have clinical consequences, as the toxicity or efficacy of *e.g*. pharmaceuticals can vary considerably between humans [36]. However, it should be noticed that an AHR binding affinity study with human placental cells did not reveal any polymorphisms that could account for the inter-individual differences in response to AHR ligands [33, 38]. This observation suggests that other, yet unidentified, (genetic) factors can account for inter-individual differences in *e.g*. CYP action.

Future prospects and applications model

The lack of (non-animal) human relevant test methods to assess female reproductive health is a clear gap in current testing strategies. The endometrial co-culture model using two types of primary human endometrial cells can be used to address an important aspect of female reproductive health: endometrial receptivity.

Key to successful reproduction, next to egg maturation and ovarian function, is embryo (blastocyst) attachment and implantation in the endometrium. The implantation of a blastocyst is dependent on the receptivity of the endometrium

that is especially influenced by estrogens and progestins [39, 40]. As described in Chapter 1, a delicate hormone balance is crucial for the implantation of a blastocyst. Any disruption of this hormonal balance by chemicals may eventually result in infertility.

Many environmental chemicals like PAHs, heavy metals, pesticides and organic solvents have been associated with a poor reproductivity outcome [41]. Especially the exposure to dioxin-like compounds is associated with infertility of both males and females [42, 43].

The 3D endometrial model with primary human cells as described in this thesis can be very useful to assess the potential effects and molecular mechanisms of (novel) chemicals on endometrial receptivity and the implantation process [47, 48].

However, it should be realized that our human endometrial co-culture model with primary cells can detect direct potential effects of chemicals on endometrial health, it does not predict indirect effects via hormonal feedback mechanisms via the HPG-axis or the interaction with other systems *e.g.* the immune system. It is challenging to mimic such interactions *in vitro*, but in the future, such feedback mechanisms may be incorporated in our human endometrial model.

4. Overall conclusion

Despite the high number of women suffering from endometrial diseases and infertility, there are still no dedicated and human-relevant assays to screen chemicals for their potential effects on female reproductive health. The human endometrial co-culture model as described in this thesis may be one useful tool to assess these effects on the endometrium. Our endometrium model also showed that there are significant differences between the human and rat endometrium that could hamper the translation of animal studies to the human situation. Cellular responses to steroid hormones and AHR ligands differ not only between rodents and humans, but also between AHR ligands and tissues. Because of these differences it remains a challenge to translate results from an *in vivo* study to the human situation with respect to female reproductive health.

The research presented in this thesis leads to the following overall conclusions:

- *The rat may not be a suitable model for human female endometrial health*
- *The use of a human endometrial co-culture model with primary cells can bridge the species differences between humans and rat.*
- *The endometrial co-culture model can lead to a better understanding of the potential endometrial health effects of chemicals to humans.*
- *The endometrial co-culture model may reduce the number of animals used in* in vivo *testing by prioritizing the number of compounds to be tested.*

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Appendix

Nederlandse samenvatting

Rationale

Ongeveer 10% van alle vrouwen in vruchtbare leeftijd wordt gediagnostiseerd met aandoeningen zoals endometriose, baarmoederkanker en verminderde vruchtbaarheid. Dit soort aandoeningen van de vrouwelijke reproductieve gezondheid komen steeds vaker voor. Deze verhoogde incidentie wordt in verband gebracht met de blootstelling aan milieuvervuilende stoffen. Het blijkt echter zeer moeilijk om deze aandoeningen te onderzoeken door de hormonale interactie tussen het brein, de eierstokken en de baarmoeder én verschillen tussen het reproductieve systeem van mensen en proefdieren. Mede door de verschillen tussen mensen en proefdieren is er een gebrek aan goede testmodellen om aandoeningen in het reproductieve systeem in mensen te onderzoeken. Het doel van dit proefschrift is het ontwikkelen van een humaan relevant endometrium model om de effecten van chemische stoffen te testen.

Achtergrond

Het vrouwelijke reproductieve systeem wordt gecontroleerd door hormonen. Voor de vrouwelijke menstruele cyclus zijn oestradiol en progesteron de belangrijkste hormonen met betrekking tot de baarmoeder. Oestradiol is het meest dominante hormoon in de eerste helft, de proliferatieve fase, van de menstruele cyclus. Hier zorgt het voor een verdikking van de binnenwand van de baarmoeder, het endometrium. Na de eisprong breekt de secretoire fase aan en is progesteron het meest dominante hormoon. In deze tweede helft van de menstruele cyclus wordt het endometrium voorbereid op een mogelijke innesteling van een bevruchte eicel. Wanneer deze innesteling niet plaatsvindt wordt het endometrium afgestoten, vindt de menstruatie plaats en begint de menstruele cyclus opnieuw.

Chemische (milieuvervuilende) stoffen of geneesmiddelen kunnen de balans tussen oestradiol en progesteron op verschillende manieren verstoren. Op deze manier kunnen ze mogelijk schadelijke gezondheidseffecten veroorzaken. De aryl hydrocarbon receptor (AHR) speelt hierbij mogelijk een belangrijke rol. De AHR is vooral bekend als sensor en regelen van afbraakprocessen van lichaamsvreemde stoffen. Echter, de laatste jaren wordt de AHR steeds meer in verband gebracht met reguleren van normale cellulaire processen. Wanneer de AHR geactiveerd wordt door chemische stoffen kan dit leiden tot een verlaagde expressie van de oestrogeen receptor, waardoor het effect van oestradiol afneemt. Ook kan dit leiden tot een verhoogde afbraak van oestradiol, waardoor de concentratie werkzame oestradiol afneemt. De gevormde oestradiol metabolieten kunnen ook interacties aan gaan met het DNA van cellen in het endometrium, met mogelijk een verhoogd risico op baarmoederkanker als gevolg.

Om het effect van chemische stoffen te kunnen voorspellen wordt vaak gebruik gemaakt van proefdieren, voornamelijk knaagdieren. Het vrouwelijke reproductieve systeem bij mensen verschilt echter aanzienlijk met dat van knaagdieren. Naast de duidelijk structurele verschillen (knaagdieren hebben een zogenoemde duplex baarmoeder voor meerdere embryo's in plaats van een baarmoeder met één kamer zoals bij mensen) zijn er ook mechanistische verschillen tussen de baarmoeder en de hormonale regulatie tussen mensen en knaagdieren.

In dit proefschrift worden een aantal verschillen tussen het endometrium van mensen en ratten onderzocht, met een speciale focus op de AHR. Ook wordt er aandacht besteed aan chemische stof-specifieke en weefsel-specifieke verschillen na activatie van de AHR.

Experimentele hoofdstukken

Om de verschillen tussen het endometrium van mensen en ratten te onderzoeken is er gebruik gemaakt van primaire endometrium cellen. Deze cellen zijn geïsoleerd zijn uit gezond baarmoederweefsel en in het laboratorium in leven gehouden. In **hoofdstuk 2** zijn deze cellen gebruikt om een 3D endometrium co-cultuur model te ontwikkelen voor zowel de mens als rat. Om de menselijke situatie zo goed mogelijk na te bootsen zijn er verschillende typen endometrium cellen gebruikt. Een celtype is in een dik laagje gel gebracht en een ander celtype is daar bovenop geplaatst. Deze 3D modellen werden vervolgens blootgesteld aan hormonen (oestradiol en progesteron) om de reproductieve cyclus na te bootsen én aan chemische stoffen welke de AHR activeren. In dit hoofdstuk zijn daarvoor een milieuvervuilende stof, tetrachlorodibenzodioxine (TCDD) en een geneesmiddel en zijn metaboliet, laquinimod en DELAQ, gebruikt. Er is gevonden dat het hormoon progesteron de expressie van de AHR verlaagt in endometriumcellen van ratten, maar niet in endometriumcellen van mensen. Ook hebben we gevonden dat DELAQ de AHR sterker activeert dan TCDD in het menselijke endometrium model, maar dat dit andersom is in het ratten model.

In **hoofdstuk 3** is onderzocht of chemische stoffen die de AHR activeren, ook het effect van oestradiol kunnen verminderen in primaire epitheliale endometrium cellen. Deze vermindering van oestrogene werking wordt ook wel anti-oestrogeniteit genoemd. Dit is een gunstig effect, wat leidt tot bescherming tegen baarmoederkanker. Alleen in de endometriumcellen van mensen is er een anti-oestrogeen effect gezien en niet in de endometrium cellen van ratten. Dit is opvallend omdat de activatie van de AHR in de endometrium cellen van ratten sterker lijkt. Ook activeerde DELAQ de AHR wederom sterker dan TCDD in de endometrium cellen van mensen, terwijl dit andersom was in de endometrium cellen van ratten, zoals ook is gezien in het 3D co-cultuur model wat is beschreven in hoofdstuk 2.

Een ander verschil in het reproductieve systeem tussen mensen en ratten is de stimulatie van progesteron productie door het hormoon prolactine wat geproduceerd wordt door de hypofyse in het brein. Als een chemische stof de potentie heeft om de productie van prolactine te beïnvloeden kan dit leiden tot verstoring van de hormonale balans bij ratten, maar niet bij mensen. In **hoofdstuk 4** is daarom het effect van AHR activatie op de prolactine genexpressie en afgifte in de hypofyse van ratten onderzocht. De productie en remming van prolactine is onderzocht in primaire hypofyse cellen van de rat en in twee veel gebruikte hypofysekanker cellijnen. We hebben gevonden dat de productie en remming van prolactine in geen van deze cellijnen overeenkomt met de normale regulatie van prolactine in een intact lichaam. Alleen de primaire hypofyse cellen reageren op stimuli waarvan bekend is dat ze prolactine produceren en remmen. Ook hebben we gezien dat DELAQ de normale prolactine remming kan beïnvloeden.

In **hoofdstuk 5 en 6** worden de weefsel-specifieke effecten van AHR activatie vergeleken door effecten in endometrium kankercellijnen en borstkanker cellijnen te vergelijken. AHR activatie bleek sterker te zijn in borstkanker cellen ten opzichte van de endometriumkanker cellen, bij dezelfde concentraties van chemische stoffen. Zelfs tussen verschillende endometrium kankercellijnen zijn verschillen in AHR activatie gevonden door activatie met chemische stoffen.

De gevonden resultaten zoals beschreven in dit proefschrift laten zien dat er grote verschillen zijn tussen het endometrium van mensen en ratten met betrekking tot hormonen maar ook met betrekking tot mogelijk toxische effecten van chemische stoffen. Hormonen verlagen de expressie van de AHR in het endometrium van ratten, maar niet in dat van mensen. Het is nog onduidelijk waardoor dit verschil veroorzaakt wordt. Wanneer dit wordt opgehelderd kan dit mogelijk helpen om toxische effecten uit dierstudies te vertalen naar de effecten bij mensen. Een tweede opvallend resultaat is de omgedraaide mate van AHR activatie tussen twee chemische stoffen die de AHR activeren in de primaire endometrium cellen van mensen en ratten. De mate van activatie van deze chemische stoffen is ook omgedraaid tussen de menselijke primaire endometrium cellen en de endometrium (kanker) cellijnen. Verschillen tussen celtypen in de aanwezigheid van co-activators en interactie met nucleaire factoren die de AHR activiteit reguleren zouden deze resultaten kunnen verklaren. Ook zou een ander AHR ligand bindingsdomein kunnen leiden tot verschillende effecten tussen soorten. Er is echter nog geen duidelijkheid om de gevonden verschillen tussen soorten, weefsels en stoffen te verklaren.

Hoe nu verder

Het 3D endometrium model met primaire endometrium cellen zoals beschreven in hoofdstuk 2 kan goed worden gebruikt om de soort-specifieke en ligand-specifieke verschillen in het endometrium te identificeren. Tevens kan het model worden ontwikkeld tot een model om het effect van chemische stoffen op de innesteling van een bevruchte eicel in het endometrium te testen. Het model kan worden gevalideerd door andere milieuvervuilende stoffen of geneesmiddelen te gebruiken en te vergelijken met proefdierstudies uit het verleden.

Om oestrogene effecten van chemische stoffen aan te tonen wordt nu vaak een proefdierstudie uitgevoerd waarbij wordt gekeken naar het gewicht van de baarmoeder. Oestrogeen stimuleert de groei van de baarmoeder in proefdieren. Chemische stoffen met een anti-oestrogene werking kunnen deze groei remmen. (Anti-)oestrogene effecten van chemische stoffen kunnen echter ook worden aangetoond met primaire cellen zoals beschreven in hoofdstuk 3. Dit levert niet alleen een vermindering van het aantal proefdieren op, maar ook een relevantere manier om dit te testen.

Conclusie

Het onderzoek beschreven in dit proefschrift bevestigt een aantal aanzienlijke verschillen tussen de reproductieve organen van mensen en ratten. Deze verschillen maken het moeilijk om een goede vertaling te maken van proefdierstudies naar een menselijke situatie. Om deze proefdierstudies beter te interpreteren is het identificeren van de verschillen tussen mens en knaagdier een cruciale stap. Alles bij elkaar genomen kan worden geconcludeerd dat een rat geen relevant model is om het effect van stoffen op de vrouwelijke reproductieve gezondheid te voorspellen. Dit komt door verschillen in hormonale respons, maar ook door soort-specifieke en chemische stof-specifieke verschillen na het activeren van de AHR. Om de effecten van chemische stoffen op het endometrium te testen kan er dus beter gebruik gemaakt worden van (primaire) humane endometriumcellen.

Dankwoord

Nothing happens unless first we dream
Carl Sandburg

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Curriculum vitae

Annick van den Brand was born on February 17th 1990 in Apeldoorn. She attended the Linde College secondary school in Wolvega, resulting in the atheneum diploma in Nature and Health. In 2008 she commenced her education by studying the Bachelor Biology at Utrecht University. She finalized this Bachelor with a thesis on the effects of endocrine disruptors and precocious puberty in girls, supervised by Prof.dr. Martin van den Berg. After a short period of volunteering in the inlands of Surinam, she started the Master Toxicology and Environmental Health with a focus on Endocrine toxicology. During this master she completed two internships; within the Endocrine toxicology group of the Institute for Risk Assessment Sciences, supervised by Prof.dr. Majorie van Duursen and within Shell Health/ the Innovative *C. elegans* testing department at the HAN BioCentre supervised by Dr. Marjoke Heneweer and Dr. Marjolein Wildwater. Her career started off at Catalyze where she wrote and managed grant proposals for other researchers. The desire of performing her own research remained and she was therefore not hesitant to accept the position as junior researcher at the Institute for Risk Assessment Sciences where she was working on short projects that eventually resulted in current thesis. As of March 2019 she works at the department for Food Safety at the National Institute for Public Health and the Environment (RIVM).

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Ad finem fidelis