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The Effects of Estradiol and Progesterone on Cytochrome P-450 (CYP) 1A1 and CYP 1B1 Enzyme Activity in Immortalized Mink Uterine Epithelial Cells

By

Joshua Tol Astle

A Thesis Submitted in Partial Fulfillment
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To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Joshua Tol Astle find it satisfactory and recommend that it be accepted.

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LIST OF ABBREVIATIONS

2Fl-E2: Two-fluoroestradiol-17β

2OHE2: 2-hydroxy estradiol

4OHE2: 4-hydroxy estradiol

4OH-TAM: 4-hydroxytamoxifen

AhR: Aryl hydrocarbon receptor

cbg: cortisol binding globulin

CL: corpus luteum

CYP: cytochrome P450

CYP1A1: cytochrome P450 1A1

CYP1B1: cytochrome P450 1B1

E2: estradiol

EM1: human endometrial glandular cell line

ER: estrogen receptor

ERα: estrogen receptor alpha

ERAF2: estrogen receptor alpha activating factor 2

ERβ: estrogen receptor beta

ERKOα: estrogen receptor alpha knockout

FSH: follicular stimulating hormone

GE: Glandular epithelium

GMMe: immortalized mink epithelial cells

hsp: heat shock protein

LE: Luminal epithelium

LF: Lactoferrin

LH: luteinizing hormone

MCF-7: Human breast adenocarcinoma cells

mRNA: messenger ribonucleic acid

Muc-1: mucin glycoprotein-1

P4: progesterone

PR: progesterone receptor

PRα: progesterone receptor alpha

PRβ: progesterone receptor beta

PRKO: progesterone receptor alpha knockout

Prl: prolactin

Shbp: sex hormone binding protein

WT: wild type

ZP: zona pellucida

ABSTRACT

Mink exhibit obligatory embryonic diapause with blastocysts in a state of arrested development for up to 60 days resulting in delayed implantation. Estradiol (E2) and progesterone (P4) are requisite to the termination of diapause, although the molecular mechanisms are unknown. We show that immortalized mink uterine epithelial cells express Cytochrome P-450 (CYP) 1A1 that synthesizes 2-hydroxyestradiol (2OHE2) and CYP1B1 that produces 4-hydroxyestradiol (4OHE2); collectively referred to as 2/4 hydroxylase. Exogenous E2 increased 2/4 hydroxylase activity in a dose dependent manner, whereas P4 inhibited the stimulatory effects of E2. We propose that in vivo, E2 increases the production of 4-OHE2, which activates the dormant mink blastocyst. Subsequently, as P4 levels rise, inhibiting CYP1B1 activity, the uterus is transformed from an estrogen-dominate proliferative phase to a secretory receptive state for embryo implantation. It is possible that P4 may in part bring to an end of the window of implantation in this species.

INTRODUCTION

Reproductive success may in general, be defined as the passing of genes onto the next generation, such that the offspring are fertile and capable of passing their genes on to their progeny (Carl Bergstrom and Dugatkin, 2012). Although reproductive failures may result from the egg not being fertilized, or the embryo not developing, it is failure of the embryo to implant in the uterine endometrium that is responsible for most pregnancy losses. Indeed, implantation failure has been estimated to be responsible for 70% of the pregnancy losses in cattle (Diskin and Morris, 2008), 60% in horses (Morris and Allen, 2002), and 75% in humans (Norwitz et al., 2001). Thus, a better understanding of the physiological mechanisms controlling embryo implantation may lead to improved domestic animal production, cures for human infertility, and perhaps safer and more effective forms of contraception.

Successful implantation depends upon the synchronized development of the embryo (blastocyst) to a state of implantation competency and a receptive uterus; collectively referred to as the window of implantation (Dey et al., 2004; Paria et al., 2002, 1998). In most mammals, implantation takes place in response to the sequential actions of estradiol (E2) and progesterone (P4) on the embryo and uterus (Dai et al., 2003; Fenelon et al., 2014; Paria et al., 1993; Torbit and Weitlauf, 1975). During follicular development, ovarian E2 promotes proliferation of the uterine endometrium, and increases P4 receptor concentrations. Subsequently, during the luteal phase, P4 decreases the proliferative actions of E2 and transforms the endometrium into a secretory tissue. The survival, development and activation of the embryo preceding implantation is dependent upon uterine secretions referred to as histotroph, containing hormones, growth factors, ions and

nutrients. Although the pre-implantation surge in E2 secretion is requisite to implantation in the rodent, the underlying mechanisms by which E2 and P4 promote development and activation of the embryo to the competent state for implantation remain largely unknown.

Hoversland et al. (1982) induced a state of delayed implantation in mice by ovariectomizing the animals on day 4 (day 1=vaginal plug) of pregnancy and maintaining them with P4 injections from days 5-7. Treatment of these mice with catechol estrogen metabolites of E2 (4-hydroxycatecholestradiol; 4OHE2; 25-50 ng/animal) and 2OHE2 (2,000 ng/animal) induced implantation. Subsequently, Kantor et al. (1985) treated hypophysectomized delayed implanting rats with 4OHE2 (with single injection or by infusion with osmotic mini pump for 24h) and induced implantation at approximately the same rates as mice receiving E2. The infusion of 2OHE2 at 50ng/h failed to induce implantation and even when infused at 200ng/h, implantation reactions were detected in only 50% of the animals.

Paria et al. (1998), in a seminal study, demonstrated that while E2 acted directly on the mouse uterus to prepare the organ for implantation, it was the metabolism of E2 by the uterus, to 4OHE2 that was responsible for blastocyst activation. Delayed implantation was induced by ovariectomizing mice on day 4 (day 1=vaginal plug) of pregnancy or pseudopregnancy and treating them with P4 injections from days 5-7. The resulting dormant blastocysts when flushed from the uteri and incubated with 4OHE2 *in vitro* became active (as judged by epidermal growth factor binding) and when transplanted into appropriately primed females, implanted resulting in live births. None of the embryos incubated with E2 were activated and none underwent implantation. It is also noteworthy that although the mouse blastocyst expresses both estrogen receptors α and β (Das et al.,

1997; Hou et al., 1996) the activating effects of 4OHE2 were not blocked by concomitant administration of the pure estrogen receptor antagonist ICN-182,780, suggesting that 4OHE2 may act through a signaling pathway distinct from the classical nuclear estrogen receptor (Paria et al., 1998).

Catechol estrogens are biologically active steroid metabolites formed through the aromatic hydroxylation of estrogens at the C-2 or C-4 positions (Zhu and Conney, 1998; Zhu and Lee, 2005). Metabolism of E2 to 4OHE2 is catalyzed by cytochrome P450 1B1 (CYP1B1) while production of 2OHE2 is mediated by CYP1A1. The high metabolic clearance rate of catechol estrogens, implies that they function as autocrine, paracrine and intracrine mediators of the effects of E2 and not as conventional circulating hormones (Ball et al., 1983; Markides and Liehr, 2005; Raftogianis et al., 2000). The biological activity of 4OHE2 is greater than that of 2OHE2. Moreover, Paria et al. (1990) showed that the production of 4OHE2 by the mouse uterus was 90% greater than 2OHE2 on day 4 of pregnancy. Finally, treatment of ovariectomized mink with 4OHE2 and 2OHE2 increased uterine epithelial cell glycogen content (Rose et al., 2011), suggesting to us that, in addition to actions on the blastocyst (Paria et al., 1998), catechol estrogens may also regulate uterine physiology in mink.

The uteri of mice (Paria et al., 1990) rats (Rataj et al., 2012) humans (Tsuchiya et al., 2004) and mink (Hunt et al., 2012) express CYP1B1. In mink, expression of the CYP1B1 protein was expressed predominantly by the glandular and luminal epithelial cells of the uterus, with reduced levels of expression by the stroma (Figure 7, Hunt, 2012). Similarly, Tsuchiya et al., (2004) showed that human uterine glandular epithelial expressed the CYP1B1 protein, that was highest during the proliferative phase of the

menstrual cycle. The expression of CYP1A1 protein was easily detected in estrus mink but was virtually undetectable during diapause and pregnancy. In contrast, expression of CYP1B1 mRNA by pregnant mink uterine homogenates was higher during the peri-implantation period (diapause) than during estrus (Figure 1, Hunt et al., 2014 Personal communication). In agreement with our findings, Paria et al. (1990) showed that CYP1B1 activity (as determined by 4OHE2 production) in pregnant mice increased significantly during the afternoon of day 4, just prior to implantation. Because the E2 surge at this time is essential to implantation in the mouse, it was proposed that the metabolism of E2 to 4OHE2 may be the trigger for implantation in this species. Moreover, treatment of ovariectomized mice with E2 resulted in a 100% increase in 4OHE2 production by uterine homogenates, only 24 h after the last E2 injection, suggesting that CYP1B1 expression and/or activity was regulated by E2. In agreement with this observation Williams-Brown et al., (2011) showed that treatment of immortalized human uterine epithelial cells with E2 increased CYP1B1 mRNA expression.

It is unknown if catechol estrogens mediate the effects of E2 on implantation in other species, especially those exhibiting obligatory embryonic diapause such as the mink (Neovison vison). Female mink may contain embryos as blastocysts in a state of dormancy (diapause) for up to 60 days resulting in a delay in implantation, (Enders, 1952; Hansson, 1947; Sundqvist et al., 1989). Although both E2 and P4 are thought to be responsible for embryonic and uterine development through implantation in mink, all attempts to terminate embryonic diapause with exogenous E2 and P4, alone and in

combination have failed (Hansson, 1947; Cochrane & Shackelford, 1962; Murphy et al., 1982,1983; Murphy 2012; Lopes et al., 2004).

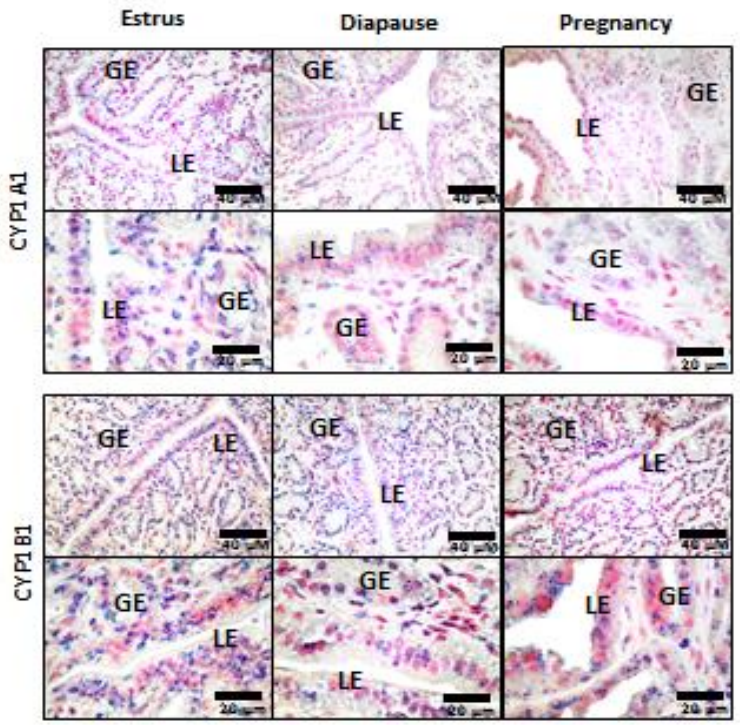

Figure 1 Immunohistochemical detection of CYP1A1 and CYP1B1 proteins in the mink uterine luminal epithelium (LE) and glandular epithelium (GE) during estrus (prior to mating); 3/11, embryonic diapause (3/23) and pregnancy (4/19; Courtesy of J.L. Hunt at Brigham Young University 2014).

Circulating E2 levels in mink increase significantly after mating, then decline and exhibit a second surge during the peri-implantation period or window of implantation (Lagerkvist et al., 1992). The concentrations of P4 begin to increase 5-10 days before implantation, peaking during mid-pregnancy, after which they decline. It is possible that treating mink with E2 to terminate diapause at an earlier time was ineffective because 4OHE2 production by the uterus was not elevated. If mink require the production of 4OHE2 by the uterus to re-activate the dormant blastocyst, it is possible that the dose and/or temporal pattern, in which the exogenous hormone was given, did not induce

CYP1B1 expression. Finally, the effects of P4 on CYP1B1 expression and activity by uterine tissue, if any, are unknown. Therefore our objectives were to determine if E2 and P4 alone, and in response to P4 following E2-priming, influence the collective activities of CYP1A1 & CYP1B1 (2/4 hydroxylase) in immortalized mink uterine epithelial cells (GMMe).

LITERATURE REVIEW

Endocrinology of the ovarian hormones Estradiol (E2) & Progesterone (P4)

Estradiol (E2): Estradiol (E2) is a steroid hormone synthesized from cholesterol. The ovaries are the primary source of E2 with smaller amounts being produced by the adrenal glands, testes, and adipose tissue (Leon Speroff et al., 1999; White and Porterfield, 2013). During the follicular phase of the ovarian cycle, Follicular stimulating hormone (FSH) stimulates E2 synthesis which peaks shortly before ovulation. After ovulation, circulating E2 concentrations decrease (White and Porterfield, 2013) as the vacant follicle luteinizes and produces progesterone (P4). Thus circulating E2 levels are highest during the follicular phase, and lowest in the luteal phase of the ovarian cycle. In some species (rodents and mink) circulating E2 concentrations increase during the window of implantation concomitantly with gradually rising P4 levels (Lagerkvist et al., 1992; McCormack & Greenwald, 1974).

The half-life of E2 in the circulation is approximately 161 minutes (Ginsburg et al., 1998, p. 45-48). This relatively long half-life is in part the result of E2 binding to proteins such as sex hormone binding protein (shbp), albumin and others, which reduces the metabolic clearance of the hormone. Estrogens are eliminated from the body in part through their conjugation with glucouronic acid and sulfates in the liver, kidneys and intestinal mucosa (Clarke and Burchell, 1994; Leon Speroff et al., 1999; Millington, 1975; Raftogianis et al., 2000). The conjugated steroids are rendered water soluble and as a result, excreted from the body via bile or urine. Interestingly, deconjugation in some

tissues may occur, promoting localized actions of the steroids (Coughtrie et al., 1998; Hobkirk, 1985).

There are two principal forms of the estrogen receptor that bind E2, referred to as ERα and ERβ (also labeled as ER-1 and ER-2). Both receptors contain DNA binding domains (96% homologous to each other) and hormone binding domains (58% homologous: Mosselman et al., 1996). The major form of the receptor in uterine tissue is ERα (Couse et al., 1997). The binding of E2 to its receptor may occur in either the cytoplasm or nucleoplasm. In addition, the receptors appear to flux back and forth across the nuclear membrane in a phenomenon called nucleoplasmic shuttling. Nucleoplasmic shuttling is defined as the continual diffusion and reuptake of a protein (in this case, the estrogen receptor) into and out of the nucleus (Dauvois et al., 1993). If this shuttling is impaired, degradation of the ERs in the cytoplasm increases sharply, reducing the estrogenic effects of the hormone (Long and Nephew, 2006).

Both receptor monomers are held in an inactive state by being bound to a heatshock protein until E2 binds, inducing a conformational change resulting in the dissociation of the protein from the receptor (Krust et al., 1986). To influence gene expression, two E2-ER monomers must dimerize before binding to promoter sites on the DNA. The dimerization of ERα and ERβ can form both homologous and heterologous pairs (Ogawa et al., 1998).

Progesterone (P4): Progesterone (P4) is a steroid hormone synthesized from cholesterol. The major source of P4 in mammals is the corpus luteum of the ovaries. Following ovulation, in response to Luteinizing hormone (LH), the now vacant follicle is

transformed (Luteinized) into a temporary endocrine organ, called a corpus luteum (CL). As the CL forms, the granulosa and thecal cells of the vacant follicle that had been synthesizing estrogens, now begin synthesizing primarily P4. If fertilization and pregnancy do not occur, the CL has a finite life span that is species specific, and slowly degenerates, resulting in reduced P4 secretion. In the event of fertilization, the developing embryo will begin secreting chorionic gonadotropin (CG) that will continue stimulating the CL to produce P4, until such time that the developing placenta begins to produce P4, and then the CL of pregnancy degenerates (White and Porterfield, 2013).

The half-life of P4 in the systemic circulation is approximately 5 minutes. In the circulation, P4 binds to various transport proteins (albumin, cortisol binding globulin), which, similar to E2, prolong the half-life of the hormone. Conjugation of P4, also increases water solubility of the hormone, increasing its elimination through the urine and bile. Metabolism of P4 to inactive metabolites such as pregnanes also contributes to P4 elimination. About 40.5±0.5% of the P4 metabolites are secreted in feces while the rest are excreted in urine (Wasser et al., 1994).

The receptor for P4, is found in two major forms; PRα and PRβ. The receptors are transcribed from the same gene, but activated at different promoter sites. The PRα promoter is found further downstream from the PRβ promoter. The PRs are virtually identical with the exception that PRα lacks 164 amino acids on its N-terminus when compared to PRβ (Horwitz et al., 1995; Kastner et al., 1990). After the PR's are translated, they bind to heat shock proteins which stabilize them until P4 binds. In the case of P4 however, heat shock proteins appear to facilitate the binding of P4 to the receptor (Wen et al., 1994). After P4 binds to its receptor, the monomers then dimerize

(similar to E2) and may then interact with DNA to influence gene expression (Williams and Sigler, 1998). In most tissues, PRβ up-regulates the transcription of progesterone responsive genes while PRα inhibits PRβ's activities (Wen et al., 1994). PRα also inhibits Estrogen's effects most likely by a non-competitive inhibition of a critical protein in the ER regulated pathway (Wen et al., 1994).

Hormonal Regulation of Blastocyst Implantation

The phrase "window of implantation" refers in part to the period of time in which the uterus is receptive to attachment and subsequent invasion by the blastocyst. The combination of sex hormones, cytokines and growth factors that regulate the window of implantation are species specific (Bazer et al., 2009; Leon Speroff et al., 1999). In mice, the window of implantation commences on day 4 when the appropriate concentrations of both E2 and P4 are present (Paria et al., 2002). Artificially varying the concentrations of these hormones from physiological levels proved to decrease or close the window of implantation (Ma et al., 2003).

The phenomenon of blastocyst implantation represents many collective events that take place before placentation occurs, and can be described briefly in four phases:

(1): preparation of the endometrium for implantation. This included extensive proliferation of both uterine epithelial and stromal cells. Also during this time many signals (growth factors, hormones & cytokines) are secreted into the uterine lumen that regulate blastocyst growth and development.

(2): Activation of the blastocyst and hatching (release) from the glycoprotein coat (zona pellucida) that surrounds it,

(3): Apposition and adhesion of the blastocyst to the uterine endometrium and

(4): Invasion of the embryo into the endometrium

Epithelial cells of the uterine lumen (luminal epithelium, LE) are contiguous with invaginations of the LE deep into the endometrium, in the form of uterine glands (i.e. uterine glandular epithelium, GE). The endometrium beneath the epithelium is composed of connective tissue, blood vessels and spindle shaped cells referred to as stromal cells. An extensive and very complicated chemical signaling (autocrine, paracrine and endocrine) occurs between the GE, LE and stromal cells that results in blastocyst activation and uterine receptivity for implantation (Cunha et al., 2004).

During phase 1, endometrial proliferation is regulated by chemical signaling (crosstalk) between uterine epithelial and stromal cells (Cunha et al., 2004). Proliferation of the epithelia is mediated primarily by E2, indirectly through signals produced by neighboring stromal cells (Cooke et al., 1997). As evidence, ERα epithelial knockouts still exhibited epithelial proliferation in response to exogenous E2. Stromal cell proliferation in response to E2 occurred only when both stromal and epithelial cells expressed ERα (Buchanan et al., 1999), which appears to be mediated in part, through Lactoferrin (LF) produced by the epithelia. During the window of implantation (luteal phase), uterine epithelial cell proliferation is inhibited by P4 in a manner similar to the effects of E2; that is, indirectly through neighboring stromal cells. As evidence, P4

inhibits epithelial proliferation even if PRα is knocked out in epithelial cells (Kurita et al., 1998; Ohta et al., 1993).

In phase 2, the blastocyst is activated, exhibiting increased mitosis and protein synthesis, ultimately leading to its "escape" or hatching from the glycoprotein coat called the zona pellucida. The zona pellucida is formed in part from secretions of the oviduct after the ovulated egg enters the fimbriated portion of the tube. At that time the zona pellucida is responsible for sperm recognition, and attachment (Florman et al., 2008; Leguia et al., 2006; Lyng and Shur, 2007), and inhibition of more than one sperm fertilizing the egg (Bleil and Wassarman, 1980; Leguia et al., 2006; Miller et al., 1993; Zhao et al., 1992). The zona pellucida is also suspected to prevent the pre-embryo from attaching to the oviduct, which would result in ectopic pregnancy (Carson et al., 2000; Daniel, 1967; Gilbert, 2010; Leon Speroff et al., 1999).

As blastocyst activity increases, the developing outer layer (trophoectoderm) extends finger-like projections into the zona pellucida and concomitantly secretes trypsin-like proteases which digest the zona pellucida enough to form a hole through which the blastocyst can escape (Gonzales et al., 1996; Perona and Wassarman, 1986).

In phase 3, apposition and adherence of the blastocyst to the uterine wall has three different stages. These stages are the removal of adhesion inhibiting polysaccharide chains called mucin (Muc-1; Hoffman et al., 1998; Meseguer et al., 2001), a period of labile attachment, followed by a period of more stable attachment (Gilbert, 2010; Wang and Dey, 2006). Once attached, the trophoblast, releases proteinases that digest the endometrial extracellular matrices, enabling the blastocyst to invade the uterine wall

during phase 4 and begin forming a placenta (Brenner et al., 1989; Gilbert, 2010; Pollheimer et al., 2006).

Blastocyst Activation: A critical event in Implantation

Although E2 and P4 are both thought to be responsible for the induction of implantation in mammals, the underlying molecular mechanisms remain largely unknown. For example, in rodents, a transient increase in circulating E2 concentrations during the peri-implantation period, is absolutely essential, without which blastocyst activation and implantation fails.

In an early study to address the phenomenon of implantation, Hoversland et al. (1982) induced a state of delayed implantation in mice by ovariectomizing the animals on day 4 (day 1=vaginal plug) of pregnancy and maintaining them with P4 injections from days 5-7. Treatment of these mice with catechol estrogen metabolites of E2 (4-hydroxycatecholestradiol; 4OHE2; 25-50 ng/animal) and 2OHE2 (2,000 ng/animal) induced implantation. Subsequently, Kantor et al., (1985) treated hypophysectomized delayed implanting rats with 4OHE2 (with single injection or by infusion with osmotic mini pump for 24h) and induced implantation at approximately the same rates as mice receiving E2. The infusion of 2OHE2 at 50ng/h failed to induce implantation and even when infused at 200ng/h, implantation reactions were detected in only 50% of the animals.

Catechol estrogens are biologically active steroid metabolites formed through the aromatic hydroxylation of estrogens at the C-2 or C-4 positions (Zhu and Conney, 1998; Zhu and Lee, 2005). Metabolism of E2 to 4OHE2 is catalyzed by cytochrome P450 1B1

(CYP1B1) while production of 2OHE2 is mediated by CYP1A1. The high metabolic clearance rate of catechol estrogens, implies that they function as autocrine, paracrine and intracrine mediators of the effects of E2 and not as conventional circulating hormones (Ball et al., 1983; Markides and Liehr, 2005; Raftogianis et al., 2000). The biological activity of 4OHE2 is greater than that of 2OHE2. Moreover, Paria et al., (1990) showed that the production of 4OHE2 by the mouse uterus was 90% greater than 2OHE2 on day four of pregnancy. Finally, treatment of ovariectomized mink with 4OHE2 and 2OHE2 increased uterine epithelial cell glycogen content (Rose et al., 2011), suggesting to us that, in addition to actions on the blastocyst (Paria et al., 1998), catechol estrogens may also regulate uterine function in mink.

The uteri of mice (Paria et al., 1990), rats (Rataj et al., 2012), humans (Tsuchiya et al., 2004) and mink (Hunt et al., 2012 MS thesis) express CYP1B1. In mink, expression of the CYP1B1 protein was expressed predominantly by the glandular and luminal epithelial cells of the uterus, with reduced levels of expression by the stroma (Hunt et al., 2014, personal communication). Expression of CYP1B1 protein was high during estrus and diapause and reduced during pregnancy. The amount of CYP1A1 protein was high in estrus but almost undetectable during diapause and pregnancy (figure 1).

Tsuchiya et al., (2004) showed that human uterine glandular epithelial expressed the CYP1B1 protein, that was highest during the proliferative phase of the menstrual cycle. Expression of CYP1B1 mRNA by pregnant mink uterine homogenates was higher during the peri-implantation period than during estrus. In agreement with our findings, Paria et al., (1990) showed that CYP1B1 activity (as determined by 4OHE2 production) in pregnant mice increased significantly during the afternoon of day 4, just prior to

implantation. Because the E2 surge at this time is essential to implantation in the mouse, it was proposed that the metabolism of E2 to 4OHE2 may be the trigger for implantation in this species. Moreover, treatment of ovariectomized mice with E2 resulted in a 100% increase in 4OHE2 production by uterine homogenates, only 24 h after the last E2 injection, suggesting that CYP1B1 expression and/or activity was regulated by E2. In agreement with this observation Williams-Brown et al., (2011) showed that treatment of immortalized human uterine epithelial cells with E2 increased CYP1B1 mRNA expression.

Paria et al., (1998) in a seminal study determined in part how E2 activates the blastocyst resulting in implantation. These investigators demonstrated that while E2 acted directly on the mouse uterus to prepare the organ for implantation, it was the metabolism of E2 by the uterus to 4OHE2 that was responsible for blastocyst activation. Delayed implantation was induced by ovariectomizing mice on day four (day one=vaginal plug) of pregnancy or pseudopregnancy and treating them with P4 injections from days five through seven. The resulting dormant blastocysts when flushed from the uteri and incubated with 4OHE2 *in vitro* became active (as judged by epidermal growth factor binding) and when transplanted into appropriately primed females, implanted resulting in live births. None of the embryos incubated with E2 were activated and none underwent implantation. It is also noteworthy that although the mouse blastocyst expresses both estrogen receptors α and β (Das et al., 1997; Hou et al., 1996) the activating effects of 4OHE2 were not blocked by concomitant administration of the pure estrogen receptor antagonist ICN-182,780, suggesting that 4OHE2 may act through a signaling pathway distinct from the classical nuclear estrogen receptor (Paria et al., 1998).

It is unknown if catechol estrogens mediate the effects of E2 on implantation in other species, especially those exhibiting obligatory embryonic diapause such as the mink (Neovison vison). Female mink may contain embryos as blastocysts in a state of dormancy (diapause) for up to 50 days or more resulting in a delay in implantation (Enders, 1952; Hansson, 1947; Sundqvist et al., 1989). Although both E2 and P4 are thought to be responsible for embryonic and uterine development through implantation in mink, all attempts to terminate embryonic diapause with exogenous E2 and P4, alone and in combination have failed (Hansson, 1947; Cochrane & Shackelford, 1962; Murphy et al., 1982,1983; Murphy 2012; Lopes et al., 2004).

Circulating E2 levels in mink increase significantly after mating, then decline and exhibit a second surge during the peri-implantation period or window of implantation (Lagerkvist et al., 1992). The concentrations of P4 increase after ovulation but remain low until about 5-10 days before implantation (i.e. window of implantation) , peaking during mid-pregnancy, after which they decline. It is possible that treating mink with E2 to terminate diapause at an earlier time was ineffective because 4OHE2 production by the uterus was not elevated. If mink, like rodents, require the production of 4OHE2 by the uterus to re-activate the dormant blastocyst, it is possible that the dose and/or temporal pattern in which the exogenous hormone was given, did not induce CYP1B1 expression. Finally, the effects of P4 on CYP1B1 expression and activity by uterine tissue, if any, are unknown.

Embryonic Diapause

At least 48 mammalian species undergo embryonic diapause that results in delayed implantation (Fenelon et al., 2014; Sandell, 1990). Embryonic diapause may be the result of environmental stresses, such as lactation (Renfree and Shaw, 2000; Weichert, 1940; Zeilmaker, 1964), nutritional status (Woodroffe, 1995), temperature (Mead, 1993), or even social stresses in mice (Marois, 1982). The dormancy of such blastocyst is referred to facultative embryonic diapause. In contrast, some species such as the mink exhibit embryonic diapause during every reproductive cycle, which is referred to as obligatory embryonic diapause (Lopes et al., 2004; Fenelon et al., 2014). It is thought that obligate diapause evolved such that birth occurs at an optimal time of year for survival of the offspring.

Reproductive cycle of Female Mink

Female mink are seasonal breeders, and become sexually receptive to males in late February to mid-March as they enter estrus (Murphy and James, 1974). Ovulation is induced by copulation approximately 36-48 h later. Fertilization takes place in the oviducts approximately 12-24 h after ovulation. The zygote develops up to the blastocyst stage of embryogenesis over the next 6-8 days and then enters the uterine lumen as a dormant blastocyst (diapause) comprised of approximately 250-500 cells, encased in an intact zona pellucida. Depending on the date of fertilization, blastocysts may remain in a state of diapause for up to 60 days.

Termination of embryonic diapause in mink takes place in response to increasing day length (photoperiod) toward the end of March. Because parturition takes place in

early May, the duration of pregnancy (implantation to parturition) is approximately 30 days. Female mink exhibit three to four estrus-like cycles per breeding season. As a result, they can mate during each ovarian cycle with the same or a different male resulting in live births from the same or different males. The fertilization of eggs from different ovarian cycles is referred to as superfetation, while the fertilization of eggs from different ovarian cycles by different males is called superfecundation (Yamaguchi et al., 2004).

Changes in circulating concentrations of E2, P4 and PRL during the reproductive cycle

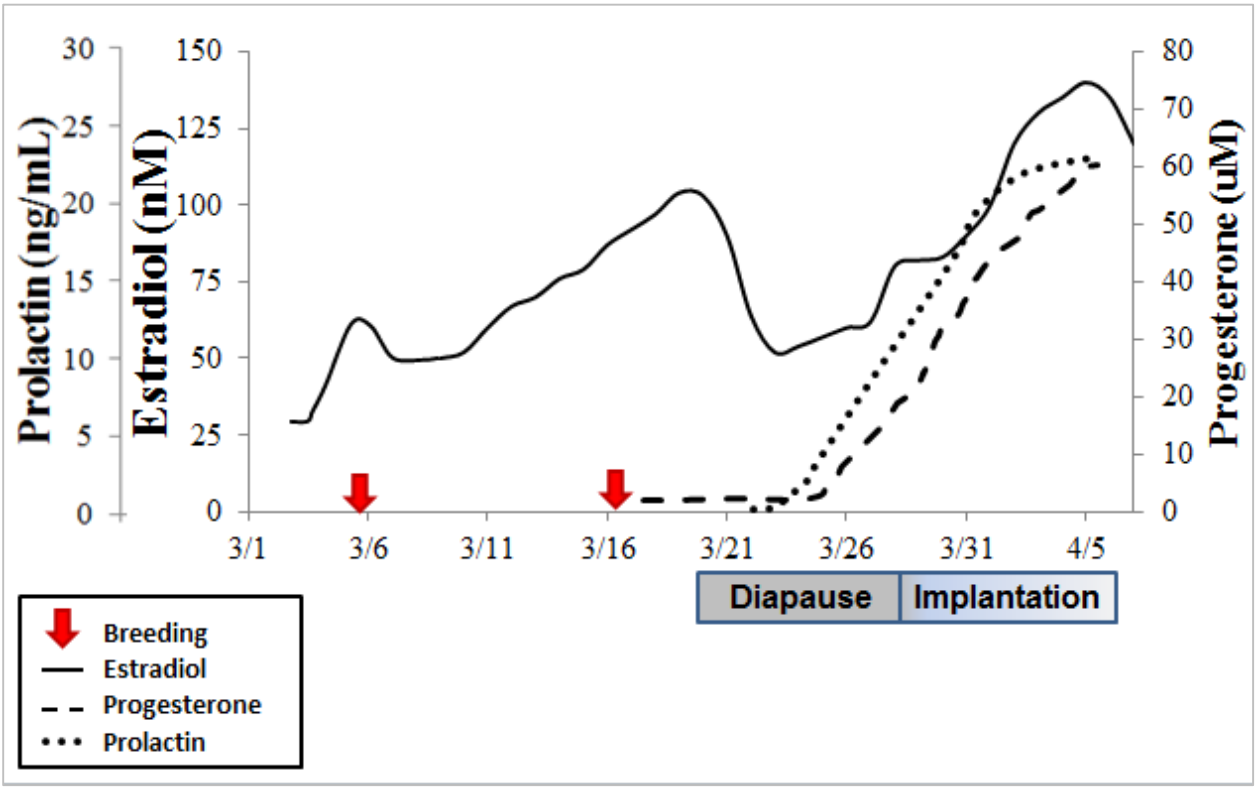

Figure 2 Circulating concentrations of estradiol (E2), progesterone (P4) and prolactin (PRL) in female mink during the reproductive season (Adapted from Lagerkvist et al., 1992; Rose et al., 1986)

In female mink, the concentrations of E2, P4 and Prl are strongly regulated by photoperiod (Murphy and James, 1974; Pilbeam et al., 1979). In the northern

hemisphere, serum E2 concentrations begin to increase in mid-February becoming very high in early March (time of estrus). After copulation there occurs a transient increase in E2, which then decreases over the next few days. During the window of implantation, E2 levels rise again (presumably from stromal ovarian tissue) and then subsequently decrease as P4 levels rise (figure 2, adapted from Lagerkvist et al., 1992). The concentrations of P4 begin to increase five to ten days before implantation (Late March-early April), peaking during mid-pregnancy (mid April), after which they decline. The sharp increase of P4 in mink around the vernal equinox is released from the reactivated corpra lutea (Allais and Martinet, 1978; Douglas et al., 1998; Lagerkvist et al., 1992; Murphy, 1983; Papke et al., 1980). The corpora lutea of mink require the actions of both LH and PRL (Murphy, 1983).

Although both E2 and P4 are thought to be responsible for embryonic and uterine development through implantation in mink, all attempts to terminate embryonic diapause with exogenous E2 and P4, alone and in combination have failed (Cochrane and Shackelford, 1962; Hansson, 1947; Lopes et al., 2004; Murphy, 2012, 1983). It is possible that uterine produced catechol estrogens (4-OHE2 and/or 2-OHE2) produced in optimal concentrations and in an optimal temporal pattern mediate the effects of E2 and/or P4 on implantation in mink.

The Effects of Estradiol and Progesterone on CYP1A1 Expression & Activity

Cytochrome P450 1A1 (CYP1A1) belongs to the cytochrome P450 super-family (Murray et al., 2001). In the presence of estradiol, CYP1A1's major product is the catechol estrogen, 2-hydroxy estradiol (2OHE2, Parl et al., 2009). The biological

potency of 2OHE2 was far less than 4OHE2 in the activation of pig blastocysts (Chakraborty et al., 1988). Rataj et al., (2012, 2014) reported that E2 down regulated CYP1A1 directly in Wistar rat uterine endometrium. Rats treated with P4 alone elicited no change in CYP1A1 activity when compared to controls but, if concomitantly incubated with E2, it tended to antagonize E2's down regulatory effects (Rataj et al., 2014).

The Effects of Estradiol and Progesterone on CYP1B1 Expression & Activity

Cytochrome P450 1B1 (CYP1B1) belongs to the same super-family as CYP1A1 (Murray et al., 2001). Expression of the CYP1B1 gene has been readily detected in human mammary gland, uterus, kidney and prostate, with weaker levels of expression in heart, brain, spleen, thymus, ovary, and colon (Shimada et al., 1996). Oncologists have been interested in the regulation of CYP1B1 expression and activity ever since it was discovered that its metabolite 4-hydroxy estradiol (4OHE2), was shown to induce tumorigenesis in Syrian hamster kidneys (Han and Liehr, 1994). Since then, scientists have explored the expression of CYP1B1 in multiple cancerous tissues with the forethought of 4OHE2's carcinogenic properties (Stack et al., 1996). A plethora of published articles report about CYP1B1 expression and activity being overly expressed in tumors and cancerous cell lines of the lung (Spivack et al., 2001), uterus (Sasaki et al., 2003a, 2003b), mammary gland (Christou et al., 1995; Parl et al., 2009; Yang et al., 2008; Yue et al., 2003), prostate (Monk et al., 2012), colon (Gibson et al., 2003), as well as skin, muscle, ovary, brain, testis, bladder, and lymph nodes (Murray et al., 1997).

Although there is much evidence to suggest that 4OHE2 may participate in tumorogenesis, there are also reports of the steroid having normal physiological actions. As stated previously, Paria et al., (1998) showed that while E2 would not activate the dormant mouse blastocyst in culture, treatment with 4OHE2 routinely activated the embryos which when transplanted into appropriately primed females, implanted and resulted in normal live births. It is therefore possible that while high concentrations of the catechol estrogen may promote abnormal growth, physiological levels of the hormone may contribute to normal homeostatic processes. It would seem that some scientists now view catechol estrogens as steroids that play important roles in normal physiological processes.

Because CYP1B1 is expressed by the uteri of mice (Paria et al., 1990), rats (Rataj et al., 2012), humans (Tsuchiya et al., 2004) and mink (Hunt et al., 2012) it is reasonable to hypothesize that the enzyme might be regulated by E2 and P4, which prepare the uterus for implantation and pregnancy. Indeed, because 4OHE2 was shown to activate the dormant mouse blastocysts for implantation, it is possible that the CYP1B1 expression and/or activity are regulated in a reproductive-cycle-dependent pattern. This may explain in part why treatment of mink with E2 and/or P4 failed to terminate diapause. That is, the treatments did not elicit the optimal amount of 4OHE2 in the correct temporal pattern.

Most evidence suggests that E2 increases CYP1B1 expression and activity through the ERα receptor pathway (Tsuchiya et al., 2004). Human breast adenocarcinoma cells (MCF-7) that were ERα positive, when treated with E2, displayed a 3-fold increase in CYP1B1 mRNA expression. In contrast, treatment of ERα negative

cells (MDA-MB-435) with E2 had no effect on CYP1B1 gene expression. Moreover, MCF-7 cells that over-expressed the ERα exhibited an increase in CYP1B1 expression of up to 80-fold in response to exogenous E2 (data not shown). Rataj et al. (2012) recently showed that treatment of rats with E2 (10ug/kg bw) for three days and killed 24 h later, exhibited a 216% increase in uterine CYP1B1 mRNA expression. These investigators treated ovariectomized Wistar rats with E2, the E2-Rα agonist 16α-LE_2 (3,17-dihydroxy-19-nor-17α-pregna-1,3,5 (10)-triene-21,16α-lactone), or the ERβ selective agonist 8β-VE_2 (8-vinylestra-1,3,5 (10)-triene-3,17β-diol) and reported that while E2 and 16α-LE_2 increased CYP1B1 expression, treatment with 8β-VE_2 decreased CYP1B1 expression. In agreement with these findings, Williams-Brown et al. (2011) provided evidence that E2 increased CYP1B1 mRNA expression in immortalized human endometrial glandular cells through ERα. Dasmahapatra et al. (2002; Comp Biochem Physiol B Biochem Mol Biol, 133; 127-134) demonstrated that CYP1B1 mRNA expression by the rat ovary increased significantly on the evening of proestrus (when circulating E2 levels are high) then decreased the morning of estrus (as E2 decreases). Collectively, these findings strongly suggest that E2 increases CYP1B1 expression and activity in a wide variety of tissues.

There are fewer reports on the effects of P4 on CYP1B1 expression and activity and to the best of our knowledge, none on uterine tissues. Christou et al. (1995) measured the CYP1B1 enzyme activity in rat mammary gland fibroblasts by measuring the concentration of 7,12-dimethylbenz[a]- anthracene (DMBA) a CYP1B1 metabolite. When cells were incubated with P4 in vitro, CYP1B1 activity was reduced when compared to controls. However, the validity of this experiment is in question since all

treatment groups were incubated with fetal bovine serum (FBS). Standard cell culture practices usually limit or remove FBS from cell cultures during experimental work since the contents of FBS can vary and also contain a myriad of hormones and other signaling molecules that could act as agonists or antagonists to treatment (Gstraunthaler, 2003). Clearly we cannot extend the findings from rat mammary gland stromal cells to uterine epithelia. However because P4 has many well-known antiestrogenic effects on the uterine epithelia such anti-proliferation as well as increased catabolism of E2 to weaker estrogens, the findings of Christou et al. would seem to be consistent with what one might expect on uterine epithelia.

OBJECTIVES

A major aim of our laboratory was to determine if immortalized mink uterine epithelial cells (GMMe) would be a useful model with which to investigate the hormonal regulation of catechol estrogen production. In collaboration with students and faculty at ISU and Brigham Young University-Idaho (Rexburg, ID), we first established using PCR and qPCR that the GMMe cells express both CYP1A1 and CYP1B1 mRNA. We then used immunohistochemistry to identify the cell types in the mink uterus that express CYP1A1 and CYP1B1 proteins during estrus, embryonic diapause and pregnancy (figure 1). The objectives of this M.S. thesis research were: (1) to develop a cell culture system to grow viable GMMe cells for experimentation, and (2) determine the effects of E2 and P4 on the activity of CYP1A1 and CYP1B1 in GMMe cells. Because the assay utilized to measure CYP enzyme activity measures both CYP1A1 and CYP1B1, we hereafter refer to the assay as the 2/4 Hydroxylase assay. We report here 2/4 hydroxylase activity in GMMe cells in response to E2 and P4 alone and in combination, as well as the effects of P4, following E2-priming.

MATERIALS AND METHODS

RNA Isolation and conversion to cDNA

Total RNA was isolated from 20-30 mg mink uterine and liver tissue using Qiagen RNeasy Fibrous Tissue Mini Kit (74104, QIAGEN, Valencia, CA, USA) as previously described (Rose et al., 2011). Samples were screened for protein contamination by measuring light absorption of each sample at 260 nm (DNA and RNA) and 280 nm (protein). Only RNA preparations with 260/280 ratio of 1.9 or greater were used for conventional PCR. To eliminate any contaminating DNA, samples were treated with genomic (gDNA) wipeout buffer prior to the reverse-transcription reaction.

Conversion of unstable RNA to stable cDNA transcripts (first strand transcripts) was achieved using QuantiTect® Reverse Transcription Kit (205311, QIAGEN, Valencia, CA, USA) according to the manufacture's instruction. The cDNA transcripts were diluted in filtered, Nano-pure water to 20 ng/mL and stored at -20^0C.

Primer Design

Since nucleotide sequences for mink target genes are unknown, we compared each target gene sequence in the rat with those of other species using the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI; NIH http:// www.ncbi.nlm.nih.gov/sites/entrez?db_genome). Regions of the rat genome for CYP1B1, CYP1A1 and CYP3A7 that were 95–100% homologous for many species (i.e., mouse, dog, and human), were used to design primers for qPCR (Table 1), which were purchased from Integrated DNA Technologies (Coralville, IA, USA).

The primer sequences used in conventional PCR were created based on DNA sequences from highly conserved regions of the CYP enzyme genes. These highly conserved regions were entered into PrimerQuest, a primer designing software created by Integrated DNA Technologies (IDT). The designed primers (table 1) were synthesized by IDT and, upon arrival to the lab, were diluted in Nano Pure water to 100uM and stored at -200C.

Table 1 Primers used for conventional PCR and each genes' accession number from which the primers were designed.

Gene*	Forward and Reverse Primers	Accession Number**
CYP1A1	GCCAGGAGAAGAGGCTA	
	TTCTGCACATTGGGGTTCGT	NM_012940.2
CYP1B1	TTGTGCCTGTCACCATTCCTC	
	TTGTCGATGAAGCCGTCCTTG	NM_012540.2
CYP3A7	CAGCCTGATACTCCTCTATCT	
	CTCACCAACACATCTCCATAC	NM_000765.4

*All genes belong to the cytochrome P450 super family.
** http://www.ncbi.nlm.nih.gov.

Conventional PCR

Although a major goal of this research was to determine CYP1B1 activity as an indication of 4-OHE2 production, the enzyme activity assay kit (Promega, P450-glo luciferin CEE assay; No.V8761 Madison WI) measures the collective activities of CYP3A7, CYP1A1 and CYP1B1. Therefore, we first analyzed the GMMe cells by conventional PCR, to determine if they expressed any or all of the CYP enzyme genes.

Conventional PCR was performed using Thermo Fischer DreamTaq Green PCR kit (# K1082) at Idaho State University's Molecular Research Core Facility in a DNA Engine Dyad® Peltier Thermal Cycler (Product numbers PTC-0220). The initial denaturation was set at 95°C for 3 min. Cycling consisted of 40 rounds. Each round was

composed of 3 different phases. These phases were denaturation (95°C for 30 seconds), annealing (54°C for 30 seconds), and extension (72°C for one minute).

The PCR products were placed in a one percent Agarose gel containing 50ng/mL Ethidium Bromide (Sigma Aldrich, #E7637) and one x TAE buffer and subjected to electrophoresis at 60 volts for three hours and the resulting blots captured using Biorad's Molecular Imager VersaDoc™ MP 4000 System (#170-8640).

DNA Sequencing

Sequencing was performed at the Idaho State University Molecular Research Core Facility, using an Applied Biosystems 3130xl Genetic Analyzer. Samples were sequenced using Applied Biosystems BigDye® Terminator v3.1 chemistry, followed by purification with Applied Biosystems BigDye® XTerminator™ Purification Kit (Applied Biosystems, #4376485).

Tissue Homogenization and Microsome isolation

In an attempt to measure CYP enzyme activity in whole organ homogenates, uteri and liver were collected from mink following sacrifice at a local mink farm in mid-November. The organs were quick frozen in liquid nitrogen and stored at -80^{o}C until assayed. At that time, mink liver (1.5 g) and uteri (n=3 for approximately 300 mg) were homogenized with a Variable-speed Stirrer (Model BDC 1850; Coframo Ltd., Ontario, CA.) on ice in 3.0 ml of homogenization buffer (100 mM KCl, 1 mM EDTA, 1 mM PMSF; Sigma Aldrich, P7626-5G, and 100mM Tris-HCl (BP153-500, Fischer Scientific, Pittsburg PA). The homogenate was aliquoted into microcentrifuge tubes and centrifuged at 10,000 g for 30 min at 4^{o}C. The resulting supernatant was then centrifuged at 100,000

g for 60 min at 4°C in a Beckman Ultracentrifuge (Model # 28-70R). The supernatant was discarded and the pellet resuspended in a 100 mM $Na(PO_4)_2$ (Fischer Scientific, # S373-500) solution. The sample was re-centrifuged at 100,000 G for 60 minutes at 4°C. This last supernatant was discarded and the resulting microsomal pellet resuspended in 3.0 ml of a storage buffer (50 mM KPO_4; Fischer Scientific, # P288-100, 0.1 mM EDTA, and 25% Sucrose) and stored at -20.0°C until assayed for CYP enzyme activity. Protein concentrations were determined for each microsomal preparation using Thermo Scientific's Comassie Plus™ Bradford assay (No. 1856210, Waltham, MA). The samples were then diluted in storage buffer to final protein concentrations of 2.0 and 4.0 mg/ml.

Culture of Immortalized Mink Uterine Epithelial Cells (GMMe)

Immortalized mink uterine epithelial cells (GMMe; CRL- 2674, American Type Culture Collection, Burlington, Ontario, CA), received frozen in growth media consisting of a one to one ratio of Dulbecco's modified eagle media (DMEM) and Ham's F12 Media (302006, American Type Culture Collection) and DMSO at 5% (v/v) were thawed in a water bath at 37°C and a 1.0 ml aliquot added to a T75 canted neck flask (430641, Corning Life Sciences, Tewksbury, MA) containing 15 mL of growth media consisting of a 1:1 ratio of DMEM, 11966-025 and Ham's F12, (11765-054; Life Technologies, Carlsbad, CA) plus FBS at 5%, 35-010-CV, Penicillin at 100 IU/ml and Streptomycin at 100 µg/mL, (30-002-Cl; Corning Life Sci.).

The flasks were then incubated for 24 hours at 37°C in a NAPCO Series 8000 WJ CO2 Incubator (Thermo Scientific) at 5% CO2. Subsequently, the cell media was pipetted off, and the cells, attached to the bottom of the plate rinsed with 6 mL filtered PBS (P5493, Sigma Aldrich, St. Louis, MO) three times to remove DMSO. Fifteen ml of

growth media was then added to the cells and returned to the incubator. After three days (and at three day intervals), the media was removed and the cells rinsed again with 6 mL filtered PBS, three times.

Cells were judged to have reached confluency (approx. 20 million cells total) at between two and three weeks. At that time the cells were washed three times each with 6.0 ml of PBS. Subsequently, 6.0 ml of a solution containing 0.05% Trypsin + 0.53 mM EDTA (Corning Life Sciences, # 25-052-CI) was added to the cells and incubated at 37°C in 5% CO_2 for 10 min to dislodge the cells from the bottom of the flask. The cells then received 27 mL of growth media (bringing the total volume to 33ml), and were mixed by flushing up and down with a pipette. This mixture is hereafter referred to as the Parent Flask.

From the parent flask, 3.0 ml aliquots of cells were transferred to the wells of six-well incubation plates (Costar, Washington DC # 3516) and returned to the incubator. After these cells reached confluence again (24-48 h), they were allowed to grow an additional 24 h. At that time, the growth media was removed, and the cells washed three times with 1 mL filtered PBS per well. After the last of the three washes was removed, the cells were then treated with hormones.

Because it was not possible to obtain enough cells for replicate measurements for all treatments from the single incubation described above, it was necessary to grow the cells to confluence repeatedly to generate the required number of cells. Thus, the Parent Flask (15.0 mls of media and cells) was returned to the incubator and allowed to grow to

confluence again. This was repeated four times resulting in the effects of hormone treatments on the GMMe cells being measured four times.

Hormone Treatments

Steroid hormones (E2 and P4) were initially dissolved in 100% dimethyl sulfoxide (DMSO; Fischer Scientific, 383 M-1733) to make stock solutions. Estradiol (E2; 026k1806; Sigma Aldrich) was dissolved at 500uM and P4 (115k0688; Sigma Aldrich) at 25 mM. Subsequent hormone dilutions were made in Treatment media consisting of DMEM + F12, and five mM glucose (A1443-001; Life Technologies) DMSO at 0.2%.

To evaluate the effects of E2 alone, the hormone was diluted from the stock solution in Treatment Media and added to wells at final concentrations of 0.1 nM, 1.0 nM, 10.0 nM, 100 nM and 1,000.0 nM or as Control (treatment media only) at time zero. The effects of P4 only, were determined by diluting the hormone from stock solution in Treatment Media and adding to each well at final concentrations of 50 uM, 40 uM, 30 uM, 20 uM, and 10 uM, or as Control (treatment media only). To simulate E2 priming before exposure to P4, we added E2 (1,000.0 nM) to each well or as Control (media only) at time zero. At 24 h, the cells were washed 3 times and re-treated with E2 at 1,000.0 nM and P4 at 50 uM and returned to the incubator for an additional 24 h, resulting in a total incubation time of 48h.

After the 48 h incubation , cells were dislodged from the bottom of the flask by adding 1 ml of a solution containing 0.05% Trypsin + 0.53 mM EDTA to each well, followed by incubation at 37°C in 5% CO2 for 15 min.

Determination of Cell Number

To ensure that CYP enzyme activity measurements did not differ because of different numbers of GMMe cells, the concentration of cells in each of the above described wells was determined as follows: A 40 ul aliquot from each well was transferred to a plastic cuvette containing 20 ml of Isoflow sheath fluid (8546859, Beckman Coulter, Brea, CA) and six drops of zap-oglobin II Lytic Reagent (7546138 Beckman Coulter) and the mixture passed through a Z2™ COULTER CELL COUNTER®, Analyzer (6605700, Beckman Coulter). Although variable, the incubations described here yielded between 800,000 and 1,600,000 GMMe cells per well. Therefore, from each well a minimum of two aliquots, in volumes calculated to contain approximately 500,000 cells were removed and centrifuged at 1,500 G for five minutes at room temp. The supernatant was discarded and 50 uLs of treatment media added to the resulting cell pellets. The pellet containing media was then removed with a pipette and placed into a well of a 96-well plate and assayed for CYP enzyme activity. This procedure was replicated four times.

The Cytochrome P450 (CYP) Enzyme Assay

The basis of CYP enzyme activity detection with the Promega kit is fascinating. Many different organisms such as fireflies, glow worms and beetles produce bioluminescence (Lee, 1976; Meyer-Rochow, 2007). Beetles produce a unique class of chromophore chemicals called luciferins. The metabolism of luciferins by luciferase results in the production of oxyluciferins, which are high-energy, peroxide intermediates. The Oxyluciferins become stable through electron transitions within the compound. It is

during these electron shifts, that a photon is released producing bioluminescence (Wood, 1995).

The Promega CYP enzyme assay kit contains pro-luciferin-CEE (luciferin 6´ chloroethyl ether), an analog of beetle luciferin. The CYP enzymes metabolize this compound to a luciferin-product, which when reacted with PROMEGA'S proprietary reagent (containing luciferase) results in luminescence. This reagent both stops CYP enzyme activity and converts the molecule to oxyluciferin, which as it undergoes stabilization, emits photons resulting in luminescence. The relative level of luminescence is an indication of the amount of CYP enzyme activity. Thus, a greater level of luminescence (enzyme activity) indicates a greater amount of CYP enzyme protein present in a biological sample.

CYP Enzyme Activity as Determined for Tissue Microsomal Preparations

Microsome preparations were thawed and re-suspended using plastic pipettes (100 ul). Subsequently, 20 uL aliquots were added to individual wells of a 96-well plate (Costar,# 3912, white wall). To each well was added 5 uLs of a Luciferin-CEE master mix (0.3 mM Pro-Luciferin Substrate; Promega, V8761), and 1 M KPO4 (Thermo Scientific, Chemical #395) and incubated for 10 min at room temp. Next Promega's NADPH regeneration system reagents were added and incubated for 30 min at room temp. Finally, 50ul of Promega's luciferin detection reagent (Promega, V8761) was added to the wells and incubated for 20 min at room temperature.

The luminescence resulting from the detection reagent was measured for each of the 96 wells using a Synergy HT plate reader (Biotek, SIAFRT, Winooski, VT) set at a

gain of 135. For each sample, luminescence values were expressed as Relative Luminescent Units (RLU), where higher RLU numbers indicate a greater amount of CYP enzyme activity. To account for background noise, the RLU value for a duplicate well containing buffer only was subtracted from control and all treatment wells. All data were then expressed as fold-changes relative to controls.

CYP Enzyme Activity of GMMe cells

To each sample of GMMe cells (500,000) transferred to a 96 well plate was added 2 uL of CYP Luciferin-glo reagent (Promega kit product #V8762 Madison WI). The plate was then covered (sealed) with Microseal 'B' Film (BioRad Catalog # MSB1001), and the plate mixed for five minutes at low speeds on a multi-purpose rotator (2309, Thermo Scientific). The plate was then removed and incubated for three hours at 37^{o}C in 5% CO2. Subsequently, 50 uL Luciferin detection reagent was added to each well and luminescence measured immediately using the Synergy HT plate reader (Biotek, SIAFRT, Winooski, VT). This process was repeated four times for each treatment. The CYP enzyme activity resulting from the various hormone treatments was normalized by subtracting from each the luminescence value of wells containing treatment media, without cells.

Statistical Analysis

All data from CYP enzyme activity experiments were analyzed by one way ANOVA with a Dunnett's post hoc test (α levels ≤ 0.05) or Tukey's post-hoc test (α levels ≤ 0.05) and, where applicable, with a simple linear regression.

RESULTS

Conventional PCR of GMMe cells

Conventional PCR analysis revealed that liver homogenates expressed all three CYP genes. Both whole mink uterine homogenates and GMMe cells expressed both CYP1A1 and CYP1B1 mRNA but not CYP3A7 mRNA. The GMMe cells expressed CYP1A1 and CYP1B1. Thus, the enzyme activity detected by GMMe cells will reflect the combined activities of CYP1A1 and CYP1B1, which hereafter will be referred to as 2/4 hyrdoxylase activity (figure 3).

Cytochrome P450 (CYP) 1A1, 1B1 and 3A7 Activity Assay in Microsomes

Mink liver homogenates exhibited an easily detected level of CYP (1A1, 1B1 & 3A7) enzyme activity that was reduced by approximately 50% when the amount of cell protein was reduced by 50%. The level of CYP (1A1 & 1B1) activity in whole mink uterine homogenates was low and not significantly different from controls (no tissue, figure 4).

CYP 1A1 and CYP1B1 Enzyme Activities in Immortalized Mink Uterine Epithelial Cells

Treatment of GMMe cells with E2 resulted in a significant increase in the collective activities of CYP1A1 and CYP1B1 at E2 concentrations of 1.0 nM and greater (figure 5). Linear regression revealed a strong correlation between dose of E2 and enzyme activity (R2=0.953). Treatment of GMMe cells with P4, tended to reduce CYP

enzyme activity at all doses, but was only significantly different from controls at 30 and 50 uM (Figure 6).

When GMMe cells were treated concomitantly with E2+P4, CYP enzyme activity was not different from controls (Figure 7). Pretreatment of GMMe cells with E2 (to simulate E2 priming) and then subjected to P4 (last 24 h) resulted in a level of enzyme activity that was significantly less than cells treated with E2 only ($P<0.05$; Figure 7). Interestingly, treatment of cells with P4 during that last 24 of a 48 h incubation resulted in CYP enzyme activity that was not different from E2 $\rightarrow$P4.

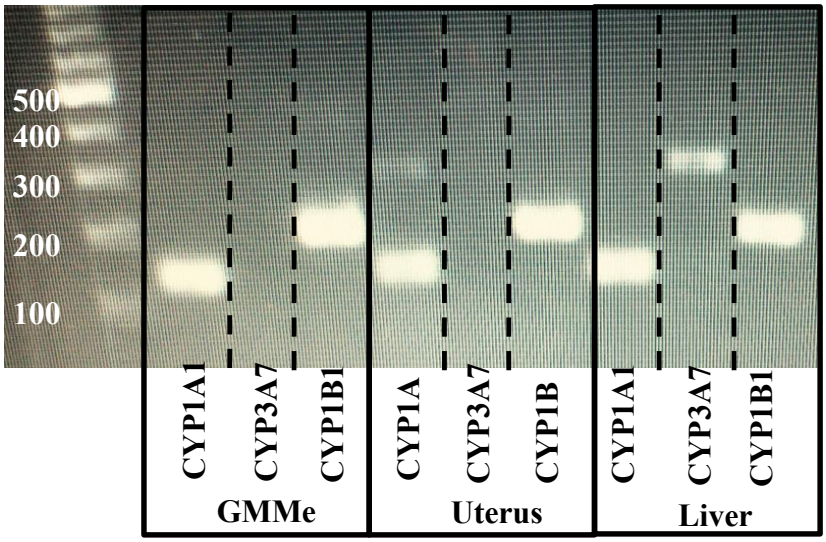

Figure 3 Expression of Cytochrome P-450 (CYP) enzyme (CYP1A1, CYP1B1 and CYP3A7) mRNA by mink uterus, liver and immortalized mink uterine epithelial cells (GMMe) The GMMe cells were grown to confluence, lysed, and DNA extracted. For whole liver and uteri, 50 mg of each were homogenized and the DNA isolated. Subsequently, all DNA were analyzed by conventional PCR.

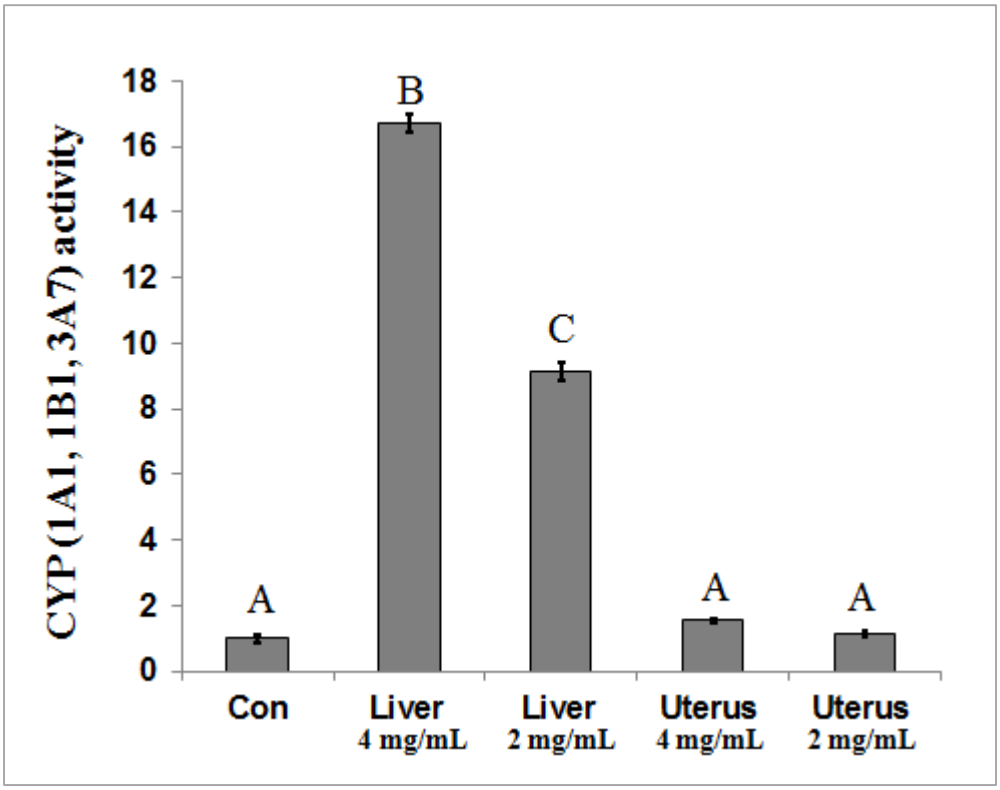

Figure 4 The collective activities of Cytochrome p-450 (CYP) 1A1, 1B1 and 3A7 enzymes in mink liver and uterine homogenates. The microsomal fraction was isolated from each and diluted to 2 and 4.0 ug protein per ul. Enzyme activity was determined using Promega kit CYP450-glo assay. Tissues that differed from one another at P=0.05, are indicated by letters (A,B &C).

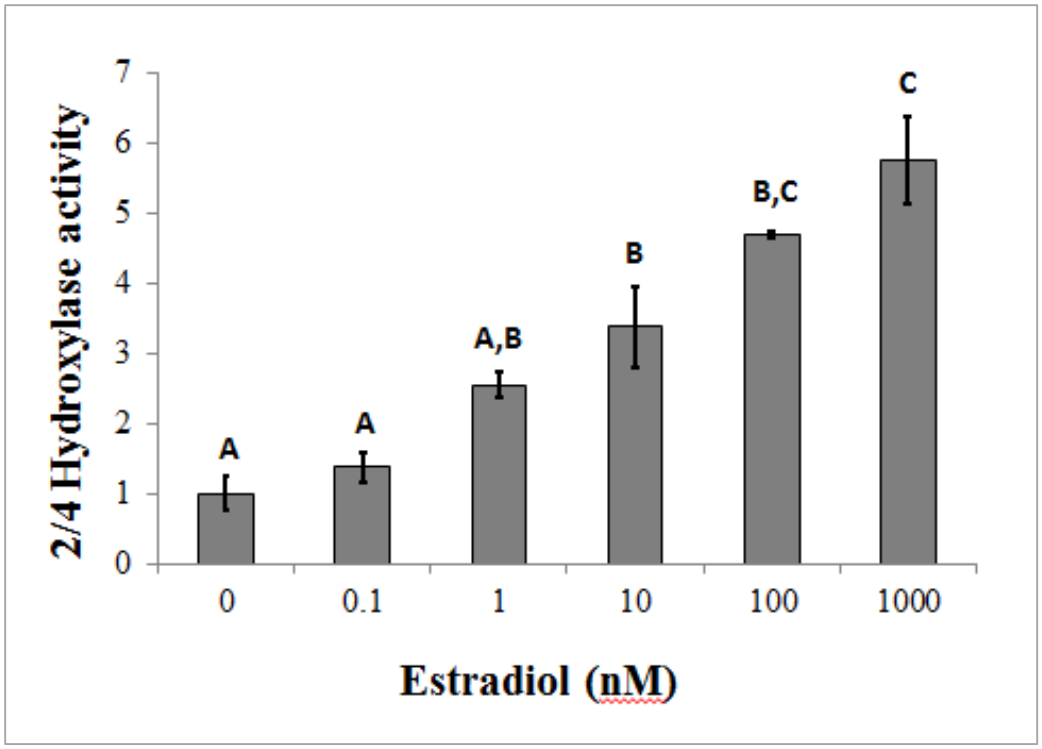

Figure 5 Collective activities of CYP1A1 + CYP1B1 in immortalized mink uterine epithelial cells (GMMe). Approximately 500,000 GMMe cells were treated with increasing concentrations of estradiol. Treatments with different letters differ from each other ($P<0.05$). Linear regression analysis revealed a high correlation between enzyme activity and estradiol concentration;
$r^2 = 0.953$

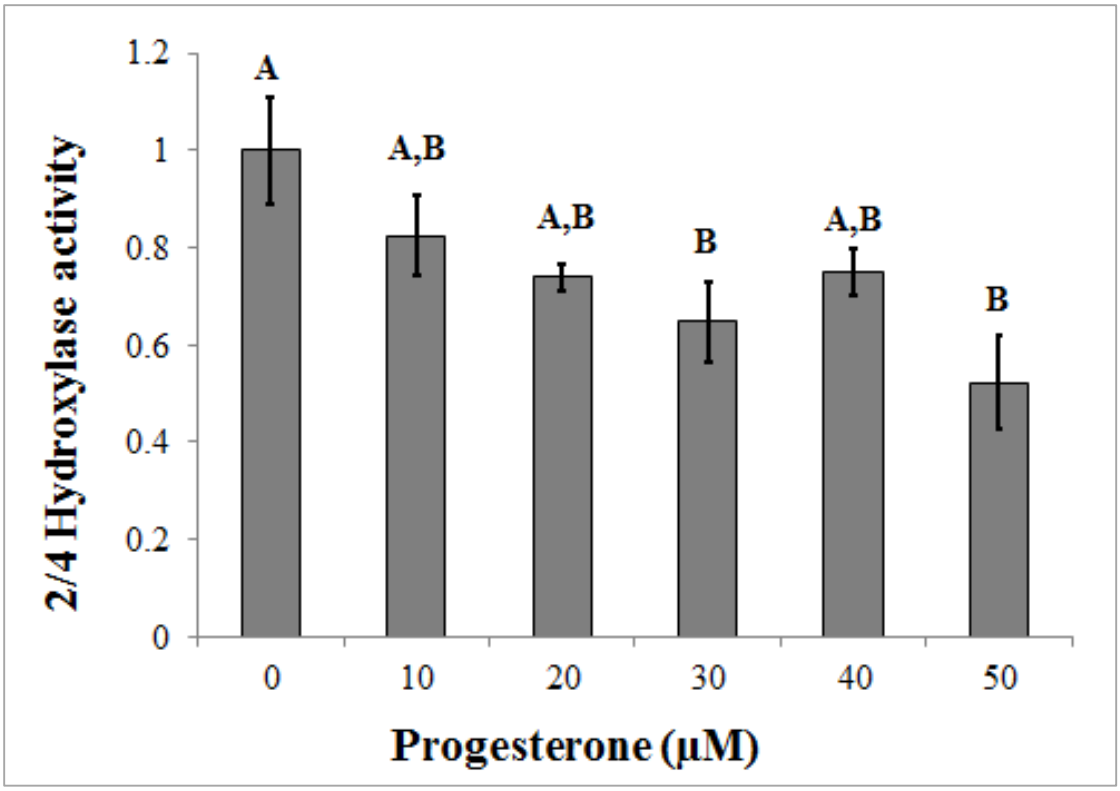

Figure 6 Collective activities of CYP1A1 + CYP1B1 in immortalized mink uterine epithelial cells (GMMe). Approximately 500,000 GMMe cells were treated with increasing concentrations of progesterone. Treatments with different letters differ from each other ($P<0.05$). Linear regression analysis revealed a low correlation between enzyme activity and progesterone concentration; $r^2 = 0.637$

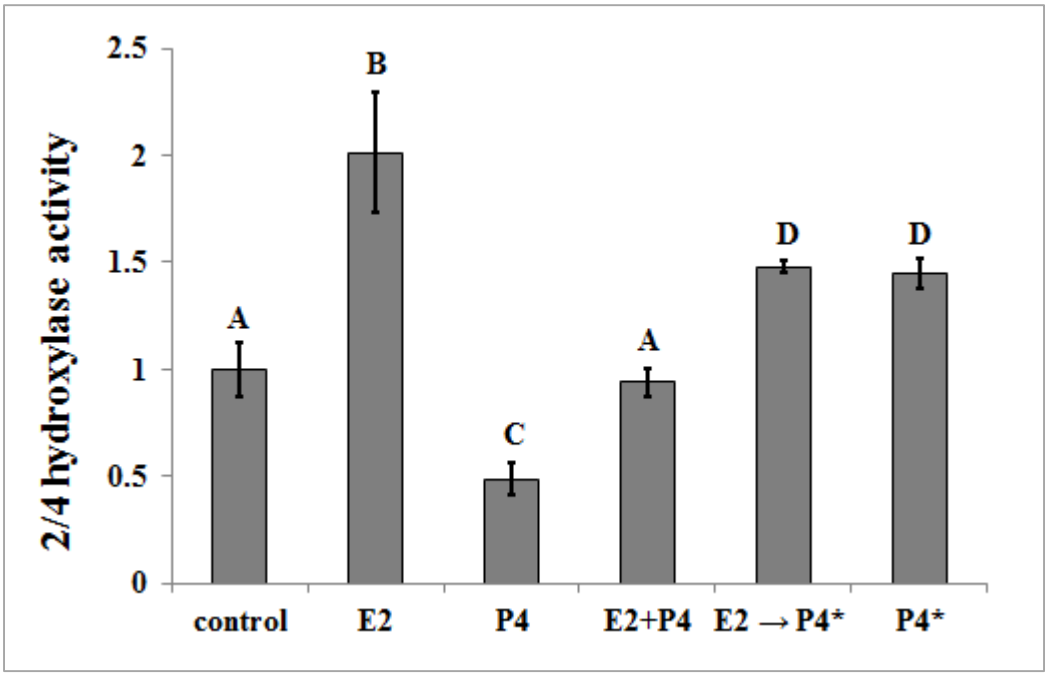

Figure 7 The effects of estradiol (E2) and progesterone (P4) alone or in response to P4 following E2-priming on Cytochrome P450 1A1 and CYP1B1 enzyme activities in immortalized mink uterine epithelial (GMme) cells. Approximately 500,000 GMMe cells were exposed either to E2 (50 μM) or P4 (1000 nm) alone or in combination for 48 h. To simulate E2 priming cells were treated with E2 for 24 h, followed by E2+P4 for the last 24 h (E2→P4*). One group of cells was treated with P4 only during the last 24 h of a 48 h incubation. Groups with different letters differ from each other ($P<0.05$).

DISCUSSION

The strong immunohistochemical signal for CYP1B1 protein in the glandular and luminal epithelia during estrus and diapause, coupled with increased CYP1B1 mRNA expression during diapause when compared to estrus (figure 1), suggests to us that uterine produced 4OHE2 may participate in reactivation of the dormant mink blastocyst. The almost undetectable level of CYP1A1 protein during diapause and pregnancy, when compared to estrus coupled with unchanging or reduced levels of CYP1A1 mRNA expression during diapause and pregnancy, lead us to conclude that 2OHE2 has little if any effects on the termination of embryonic diapause in mink. Although our 2/4 hydroxylase activity assay will not distinguish between CYP1A1 and CYP1B1, Paria et al. (1990) showed that the mouse uterus produced predominantly 4OHE2 (90%) with only minimal amounts of 2OHE2 (10%). It is therefore likely that our 2/4 hydroxylase assay is measuring primarily CYP1B1 activity.

The dose-response relationship between E2 and 2/4 hydroxylase activity in GMMe cells suggests to us that E2 stimulates the synthesis of 4OHE2 by the mink uterus in a very tightly controlled temporal pattern. It is likely that as circulating E2 concentrations increase following mating and again during the window of implantation (end of March to early April), intrauterine concentrations of 4OHE2 increase in parallel with circulating E2 concentrations. In other words, as circulating E2 levels increase, this is mirrored by elevations in intraluminal 4OHE2 concentrations, which could ultimately reactivate the mink embryo.

As circulating P4 levels increase during the window of implantation, and the ratio of E2:P4 progressively decreases, this may result in reduced 4OHE2 synthesis, and the

end of the window of implantation. We hypothesize that the critical concentration of 4OHE2 within the uterine, for implantation, is determined in part by both E2 and P4. Thus, it is likely that a transient rise in uterine 4OHE2 levels, regulated by E2 and P4 occurs concomitantly with uterine development to the receptive state results in successful embryo implantation. Although P4 inhibited 2/4 hydroxylase activity at all concentrations, responses were not significant until cells were treated with 20uM of the hormone. This suggests to us that circulating E2 levels predominate and increase 4OHE2 production, until circulating P4 concentrations reach a critical level. Then, P4 will exert classic anti-estrogen actions, which include reduced 2/4 hydroxylase activity. As evidence, mink primed with E2 and then treated with P4 (E2→P4) exhibited reduced 2/4 hydroxylase activity, that was still greater than controls (Figure 5). Thus, P4 levels may have to become significantly elevated before uterine 2/4 hydroxylase activity is inhibited. This later affect may contribute to the uterus transforming from a proliferative to a secretory receptive state for embryo implantation. Indeed, this action of P4 may in part be the signal which brings about the end of the window of implantation in this species.

Based on these findings, we feel that previous attempts to terminate diapause and induce implantation in mink with exogenous E2 and P4 may have failed because: (1) Treatment with P4 with or without E2, probably inhibited uterine 4OHE2 synthesis, (2) Treatment with E2 only may have resulted in excessive direct stimulation of the uterus and/or gave rise to toxic levels of 4OHE2, making the uterus unresponsive (non-receptive) to blastocyst invasion. As evidence, incubation of uterine glandular epithelia with high E2 concentrations caused apoptosis (Chen et al., 2014). In women who have undergone superovulation it was reported that a set of genes that were normally up-

regulated during the window of implantation, failed to increase and it was suspected that this may have contributed to reduced endometrial receptivity (Zhao et al., 1992). Fatemi and Popovic-Todorovic, (2013) showed that high E2 levels after ovarian stimulation lead to premature progesterone elevation, advancing endometrial development, out of synchrony with blastocyst development hampering implantation. Benadiva and Metzger (1994) showed that human ovarian hyper stimulation resulted in dyssynchronous development of glandular epithelium and stromal cells that might contribute to reduced uterine responsiveness. In summary, the simplest explanation for failure of exogenous E2 and/or P4 to terminate diapause in mink is failure to achieve an optimal (homeostatic) hormonal milieu, which includes E2, P4 and 4-OHE2.

SUMMARY

In summary, our findings when viewed in the context of the published literature suggest to us that the proximal signal that reactivates the dormant mink embryo resulting in implantation may be 4OHE2. Catechol estrogens have extremely short half-lives and as such, it is not surprising that a close to 1:1 relationship between circulating E2 levels and intrauterine 4OHE2 concentrations may exist, if the metabolite does serve to mediate the actions of E2. The actions of 4OHE2 would teleologically be required for only a short period of time (blastocyst activation) whereas the effects of E2 (endometrial proliferation and activation of implantation-related genes) would take place over a longer period of time. Moreover, the production of another estrogen (4OHE2), besides E2, might eliminate excessive and/or inappropriate stimulation of the uterus. As circulating P4 levels increase, the effects of this steroid may serve to both modulate the level of CYP1B1 activity and therefore intrauterine concentrations of the catechol estrogen. When P4 concentrations reach a critical level, the hormone may then through its anti-estrogen actions, which could include inhibiting CYP1B1 activity, close the window of implantation.

SUGGESTED FUTURE RESEARCH

1. Because the effects of E2 on uterine epithelial physiology (i.e., proliferation) are in some cases mediated through the neighboring stromal cells (Cooke et al., 1997; Kurita et al., 1998), it is possible that the responses of GMMe cells observed in this research might differ in the presence of mink stromal cells. Because immortalized mink uterine stromal cells are available, it should prove productive in the future to conduct experiments similar to those presented in this thesis, by examining the effects of E2 and P4 on CYP enzyme activity in the presence and absence immortalized uterine stromal cells.
2. Because the assay utilized here does not measure catecholestrogen production, experiments similar to those described here should be conducted in that cells should be treated with E2 and P4, but then the cell extracts should be analyzed by HPLC to quantify the actual amounts of 2-hydroxyestradiol and 4-hydroxyestradiol produced.
3. It has also been shown that prolactin rises around the time of implantation in mink (Rose et al., 1986). It would be wise to measure prolactin's effects on CYP1B1 and CYP1A1 enzyme activity alone and in combination with E2 and P4.

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