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Review

The significance of estradiol metabolites in human corpus luteum physiology

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ABSTRACT

The human corpus luteum (CL) is a temporary endocrine gland derived from the ovulated follicle. Its formation and limited lifespan is critical for steroid hormone production required to support menstrual cyclicity, endometrial receptivity for successful implantation, and the maintenance of early pregnancy. Endocrine and paracrine-autocrine molecular mechanisms associated with progesterone production throughout the luteal phase are critical for the development, maintenance, regression, and rescue by hCG which sustains CL function into early pregnancy. However, the signaling systems driving the regression of the primate corpus luteum in non-conception cycles are not well understood. Recently, there has been interest in the functional roles of estradiol metabolites (EMs), mostly in estrogen-producing tissues. The human CL produces a number of EMs, and it has been postulated that the EMs acting via paracrine-autocrine pathways affect angiogenesis or LH-mediated events. The present review describes advances in understanding the role of EMs in the functional lifespan and regression of the human CL in non-conception cycles.

1. Introduction

The secretion of estradiol (E_2) throughout the ovarian cycle in women depends upon follicle recruitment, selection of a single dominant follicle, followed by the LH surge that ends the program of FSH-dependent steroidogenesis in granulosa cell (GCs) and initiates the differentiation of follicular cells into granulosa lutein and theca lutein cells. The two cell, two gonadotropin model of E_2 biosynthesis in the preovulatory follicle encompasses androgens production by theca cells followed by their aromatization by P450arom in GCs of the follicle. The theca lutein and granulosa lutein cells (GLCs) of the human CL carry out similar steroidogenic functions to generate E_2 in the CL [1].

Ovarian estrogens have a variety of biological effects on different human tissues, and many of these effects are mediated by nuclear E_2 receptor (ER) [2,3]. Estradiol is metabolized by diverse metabolic pathways including hydroxylation, glucuronidation, sulfonation and methylation to form estrogen metabolites (EMs). These transformations take place mainly in the liver and specific extra-hepatic tissues e.g., (brain, kidney, ovaries, and testis). While a number of the EMs are inactive, others have important effects on the physiology of different tissues, particularly in E_2 producing tissues, acting through diverse pathways not associated with classical nuclear ER. [4,5]. It is important to emphasize that the human CL produces high levels of E_2 (leading to blood levels of 150–200 pg/ml) during mid-luteal phase [6], and expresses cytochrome P4501A1 (CYP450) and catechol-O-methyl transferase (COMT), such that luteal E_2 synthesized by GLCs could be converted into multiple EMs catechol and methoxy estrogens [7–9].

The prominent role of the human CL in the elaboration of steroid hormones requires a complex vascular network established through neovascularization after ovulation [10,11]. The capillary expansion requires endothelial cell proliferation, which is associated with a decreasing resistance index to blood flow as assessed by traditional Doppler ultrasound [12]. However, conventional Doppler does not quantify blood flow accurately through small capillary structures such as the network formed in the CL. Use of contrast reagents combined with destroy-replenishment imaging dynamic contrast-enhanced ultrasound (DCE-US) increases the accuracy of blood flow assessment and blood volume measurements in the CL of primates and confirms the low resistance index of early and mid-luteal phase CL [13]. The vascular features of human luteal regression include increased in blood flow impedance [12]. Based on these findings, and the known action of catecholestrogens, 4-hydroxyestrone (4-OHE_1) and 2-hydroxyestradiol (2-OHE_2), which are pro-angiogenic [14,15]; and methoxyestrogens, 2-methoxyestradiol (2-ME_2) and 2-methoxyestrone (2-ME_1), which are

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anti-angiogenic [16–19], we examined the potential role of EMs in the regulation of angiogenesis in the human CL.

2. Laboratory and clinical trials of the investigation

Human CL were collected at mini-laparotomy from women aged 30–33 years who requested surgical sterilization at our institution. The participants were healthy with normal body mass index an regular menstrual cycles. The surgical procedure was scheduled at varying times during the luteal phase. The day of ovulation and the histological criteria were used to confirm the age of the CL as previously reported [20].

Laboratory techniques: A biopsy of CL tissue for histology and immunohistochemistry was fixed in paraformaldehyde [21]. Another portion of CL (100 mg) was homogenized, and steroid extraction was performed with ethyl-acetate. The EMs were quantified by high performance liquid chromatography-mass spectrometry [22].

3. 4-Hydroxyestrone (4-OHE_1) and 16-ketoestradiol (16-ketoE$_2$) in the human corpus luteum throughout the luteal phase

Angiogenesis in the early CL has its origins in the vasculature of the developing follicle. Capillary expansion appears to be related to endothelial cell proliferation, and the vast majority of dividing cells in the early developing CL are microvascular endothelial cells [10,11]. It is thought that the mid-cycle LH surge increases VEGF production by the GLC in the developing CL [23]. Concomitant with these vascular changes, we found that 4-OHE_1 and 16-ketoE$_2$ concentrations in the human early and mid-luteal phase CL are significantly greater than in late luteal phase CL. Interestingly, *in vivo* administration of hCG in the late luteal phase increased significantly the luteal tissue levels of 4-OHE_1 and 16-ketoE$_2$, observations that are consistent with a paracrine role of EMs in angiogenesis or maintenance of the capillary network in the CL (Fig. 1A) [24].

To expand our understanding of pro-angiogenic activities of these EMs, we measured the VEGF concentrations in conditioned media (CM) from GLCs cultures in the presence of physiological concentrations of 4-OHE_1 and 16-ketoE$_2$, showing that both EMs increased significantly VEGF secretion by GLCs and the pro-angiogenic potential of the CM (Fig. 1B and C). These findings suggest an important role for these EMs in the formation and development of the CL [24].

It is important to mention that local factors such as insulin-like growth factors (IGF)-1 and -2 may synergize with LH to promote VEGF production [25]. However, the usual stimulator of VEGF production in most tissues is hypoxia [26]. The hypoxic microenvironment that occurs in rapidly differentiating tissues like the human CL is a major contributor to its ability to survive via the induction of an intricate vascular network. Cellular responses to hypoxia are mediated by hypoxia inducible factor-1A (HIF-1A), a heterodimer consisting of a constitutively-expressed β subunit an oxygen-regulated α subunit, which binds to the hypoxia responsive cis elements present in the promoter regions of responsive genes [27]. Several genes are critical for the angiogenic process in the developing CL: vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF-2), prokinectin receptor 2 (PK-R2), and endothelin-2 [28].

4. 2-Methoxyestradiol in the human corpus luteum throughout the luteal phase

2-Methoxyestradiol (2-ME$_2$) is a biologically active metabolite of E$_2$ that has antiangiogenic and anti-proliferative effects [16–19]. Its anti-angiogenic actions are believed to be the result of inhibition of HIF-1A action [29]. In the non-fertile cycle, the CL of the human undergoes a process of regression known as luteolysis, which encompasses loss of functional and structural integrity of the gland [30]. The molecular events involved in human luteal regression in non-conception cycle remain to be elucidated. It is thought that a decline in LH levels and LH receptor mRNA do not account for luteal regression in primates [31], but that regression of the CL is determined by factors downstream from the LH receptor. Several molecule including prostaglandin PGF2-α, TNF-α, IL-1β, estrogens, and reactive oxygen's species have been implicated in the luteolytic process [1–32]. In exploring the roles of

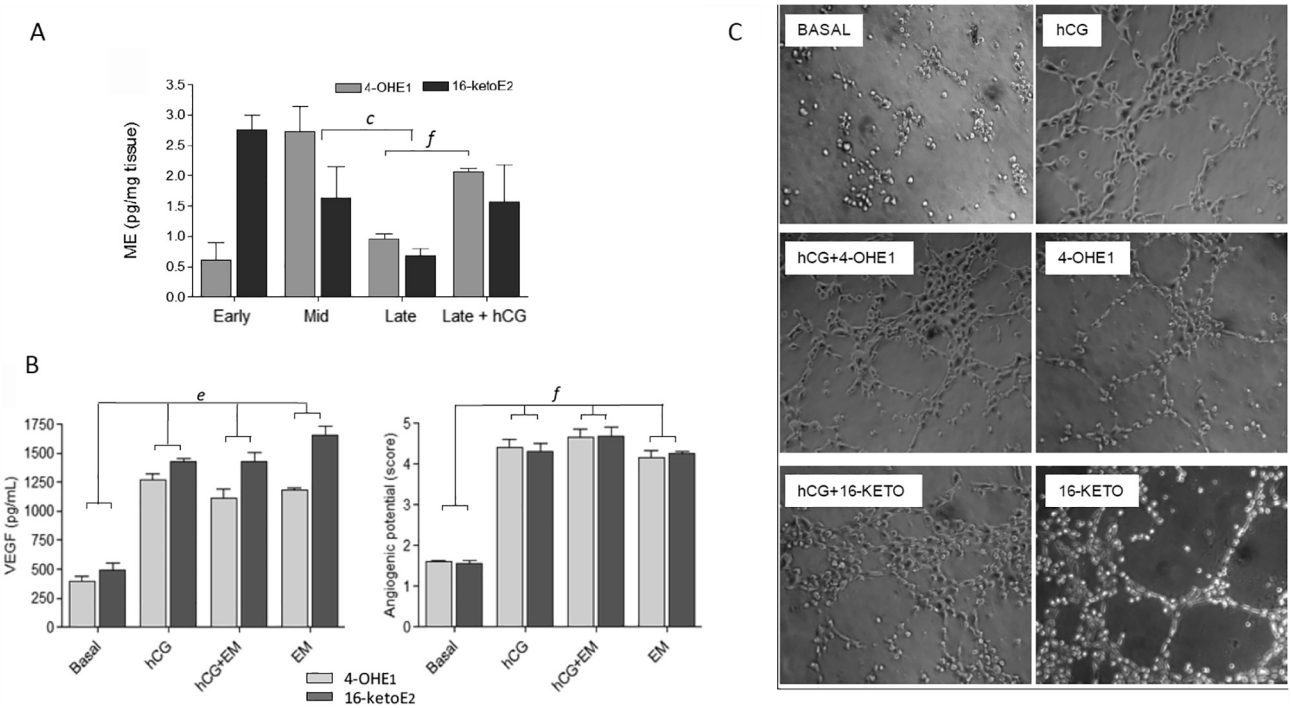

Fig. 1. Tissue levels of 16-ketoE$_2$ and 4-OHE_1 in the CL throughout the luteal phase and after administration of hCG. Effect on VEGF production and angiogenic activity by GLCs cultures. Panel A, late 16-ketoE$_2$ and 4-OHE_1 v/s mid and late plus hCG ($^{c,f}P < 0.05$) n = 5 per CL stage. (B) 16-ketoE$_2$ and 4-OHE_1 increased VEGF production by LGCs compared to basal conditions ($^{e}P < 0.05$). (C) Photomicrograph of the angiogenic assay, both EM increased tube formation compared to basal conditions ($^{f}P < 0.05$).

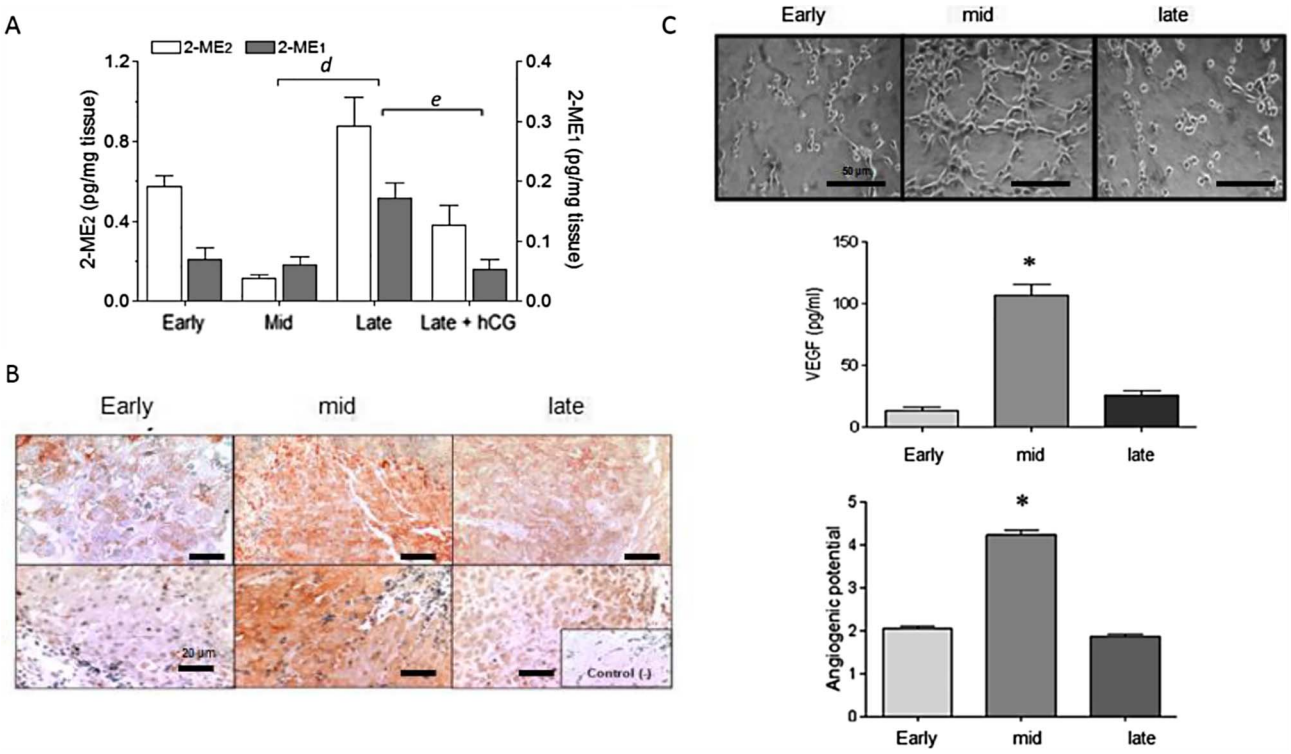

Fig. 2. Tissue levels of 2-ME_2 and 2-ME_1 in the CL throughout the luteal phase and after administration of hCG. Immunohistochemistry of VEGF and VEGF-R in CL of different ages and angiogenic factor assays. Panel A, late 2-ME_2 and 2-ME_1 v/s mid and late plus hCG ($^{d,e}P < 0.05$) n = 5 per CL stage. Panel B, The photomicrograph represents luteal tissue collected during early, mid- and late luteal phases stained for VEGF and VEGF-R, showing prominent staining in the mid-luteal phase CL compared to early and late luteal phase CL (n = 5, $P < 0.05$). Panel C, VEGF levels determined in culture media of luteal cells is greater in mid-luteal phase CL cells compared with early and late luteal phase CL cells (n = 9, $P < 0.05$). The photomicrograph represents the angiogenic assay of luteal cells culture of different ages. Conditioned media from mid-luteal phase cell cultures significantly increased the angiogenesis ($P < 0.05$).

EMs, we demonstrated that the 2-ME_2 pathway could play a role in the process of luteolysis [33]. Our findings revealed a decrease in plasma levels of E_2 in late luteal phase in association with an increase in late luteal phase tissue levels of 2-ME_2 (Fig. 2A). A cultured GC system was used to evaluate angiogenic activities and VEGF secretion in the presence and absence of 2-ME_2 in physiological concentrations. Interestingly, hCG significantly increased the angiogenic activity of conditioned media from granulosa cell cultures ($P < 0.05$), as revealed by the formation of capillary-like structures and more complex polygons, while 2-ME_2 treatment significantly reduced tube formation compared to basal conditions ($P < 0.05$). However, 2-ME_2 did not reduce the pro-angiogenic activity stimulated by hCG. Similar finding were noted in VEGF levels in conditioned medium of granulosa cells cultured in the presence of hCG, 2-ME_2 or 2-ME_2 plus hCG [33]. Our observations are consistent with the participation of 2-ME_2 in the process of luteolysis by reducing angiogenesis in the non-conception cycle. It is noteworthy that 2-ME_2 did not prevent *in vitro* hCG stimulation of P_4 biosynthesis [33].

5. Hypoxia in ovarian angiogenesis

The CL is a major site of angiogenesis and neovascularization in the early and mid-luteal phases. CL development is associated with hypoxic conditions, followed by increased levels HIF-1A and VEGF expression. Moreover, a reduction angiogenesis may play a role in luteal regression in a non-conceptional cycle. We explored some features of angiogenesis including the expression and quantitation of one of the most potent stimulators of endothelial cells proliferation (VEGF-A) and its receptor (VEGF-R) in CL of different ages (Fig. 2B) [33]. Other reports have previously described greater mRNA expression of VEGF-A in the mid-luteal phase CL [34,35]. Our data on VEGF secretion by cultured luteal cells are consistent with these findings (Fig. 2C). Interestingly, GLC cultures from other species treated with 2-ME_2 at high doses resulted in decreased VEGF production [36,37]. Other reports suggest that pig GCs cultured under restricted oxygen conditions produce increased amounts of 2-ME_2, which may explain the reduction in VEGF production *in vitro*. Although the mechanisms of luteolysis in human are not similar to the pig, [38], it is tempting to speculate that the increasing expression of HIF-1A in cells exposed to hypoxia fails to activate VEGF transcription in the presence of methoxyestrogens. It has been reported that HIF-1A inhibition by 2-ME_2 induces cell death via activation of the mitochondrial apoptotic pathway in acute myeloid leukemia cells. This research showed that expression of HIF-1A is suppressed by 2-ME_2. At the same time, 2-ME_2 downregulated the transcription of VEGF [39], consistent with the fact that HIF-1A is known to be a transcription factor that binds to the VEGF promoter, stimulating its transcription. The lower levels of VEGF diminish angiogenesis. This physiological setting of hypoxia could be present during CL regression.

6. Effect of hCG in the rescue of human corpus luteum

The endocrinologic characteristics of conception and non-conception cycles are different [40]. In human conception cycles, trophoblast production of hCG prevents regression of the human CL. Serum hCG is detectable at the time of implantation (from day 8 to day 11 post LH surge) and rises progressively during the first 12 weeks of pregnancy [40]. The CL volume rapidly increases in early pregnancy, largely due to the proliferation of non-steroidogenic cells. However, there is limited knowledge about the molecular changes underlying the functional and structural changes in the CL of pregnancy [6]. We and other investigators have employed experimental protocols including hCG administration during mid- and late luteal phases in non-conception cycles with the objective of determining the molecular basis of CL rescue. Administration of hCG during the mid- and late luteal phase restores the abundance of Steroidogenic Acute regulatory protein (StAR) mRNA and

protein levels to those found in mid-luteal phase CL as well plasma P_4 levels [41]. Additionally, hCG administration enhances the vascular networks, blood flow, relaxin secretion and decreases the apoptotic program in the late luteal phase CL [42,43]. Recently, our group determined *ex vivo* and *in vitro* effects of hCG on EM levels and their pro- or anti-angiogenic effects. hCG administration increased the production of EMs with pro-angiogenic activity (16-Ketoe$_2$ and 4-OHE$_1$) with a parallel increase in VEGF secretion, and caused a significant reduction in late luteal phase tissue of those EMs with anti-angiogenic action (2-ME$_2$ and 2-ME$_1$) (Figs. 1A and 2A). Our observations suggest novel paracrine mechanisms driven by different EMs in the development and demise of the CL during conceptional and non-conceptional cycles [24].

7. Summary and concluding remarks

The function of the CL is essential for a normal menstrual cycle and for maintenance of early pregnancy. Significant progress has been achieved in our understanding of human CL physiology and pathophysiology. This progress comprises new concepts in endocrinology, angiogenesis, and regulation of luteal steroidogenesis throughout the lifespan of the CL, including ovarian hyperstimulation syndrome. It is important to mention that the human CL secretes androgens and estradiol in addition to P_4. The unique steroidogenic futures give us the opportunity to examine the physiological roles of EMs in human CL function. One of the current outstanding issues in CL physiology is the identification and characterization of molecules that initiate the process of luteal regression in the normal menstrual cycle. The antiangiogenic and antiproliferative effects of 2-ME$_2$ in breast and endometrial cells are well known. We postulate that 2-ME$_2$ plays a role in the process of luteolysis, while 4-OHE$_1$ and 16ketoE$_2$ play roles in increasing angiogenesis in the early and mid-luteal phases of the normal menstrual cycle. Furthermore, we suggest that in a conception cycle, hCG rescues the mid- -and late luteal phase CL by increasing angiogenesis, the ability to steroid hormones, and diminishes apoptosis.

Declaration of interests

The authors have nothing to disclose.

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