

Estrogen metabolites in human corpus luteum physiology: differential effects on angiogenic activity

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Objective: To determine tissue concentrations of E₂, estrone, P, and estrogens metabolites (EMs) 2-methoxyestradiol, 2-methoxyestrone, 4-hydroxyestrone, and 16-ketoestradiol in corpus luteum (CL) of different ages, and after hCG administration; and to examine the effects of EMs on vascular endothelial growth factor (VEGF) secretion and angiogenic activity released by cultured luteinizing granulosa cells in the presence and absence of hCG.

Design: Experimental study.

Setting: University.

Patient(s): Thirty-two healthy women of reproductive age.

Intervention(s): Corpus luteum was collected at the time of minilaparotomy for tubal sterilization, at varying stages of the luteal phase (LP). Late-LP CL was collected 24 hours after IM administration of 10,000 IU hCG. Granulosa cells were isolated from follicular aspirates obtained from healthy women participating in our IVF program for male factor infertility.

Main Outcomes Measure(s): Estrogen metabolite concentrations were determined in CL tissue, and VEGF was assessed in conditioned medium. The angiogenic activity was analyzed by bioassay.

Result(s): Concentrations of EMs with proangiogenic activity (16-ketoestradiol and 4-hydroxyestrone) were higher in early and mid-LP CL vs. late-LP CL. These EMs and hCG increased VEGF production and angiogenic activity. Conversely, late-LP CL had significantly higher levels of 2-methoxyestrone and 2-methoxyestradiol, which have antiangiogenic activity. Administration of hCG reduced the production of these EMs.

Conclusion(s): Our findings suggest that the EMs are important paracrine modulators of CL function. Administration of hCG increases the production of EMs with proangiogenic activity and reduces the secretion of those EMs with antiangiogenic action, suggesting a novel mechanism by which the late-LP CL is rescued in conception cycles. (Fertil Steril® 2016;106:230–7. ©2016 by American Society for Reproductive Medicine.)

Key Words: 2-Methoxyestradiol, 2-methoxyestrone, 16-ketoestradiol, 4-hydroxyestrone, human corpora lutea

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The human corpus luteum (CL), a temporary endocrine gland derived from the ovulated follicle, is the principal source of steroid hormones during the secretory phase of the human menstrual cycle. It produces

a significant amount of P, androgens, and E₂. After ovulation, LH pulses play a central role sustaining CL steroidogenic function. A prominent characteristic of early and mid-luteal phase CL is the establishment of a rich vascular network. This vascular development facilitates access of lipoproteins that provide the cholesterol substrate for steroid biosynthesis by luteinized granulosa and theca cells. Estradiol biosynthesis is dependent upon androgens production by theca

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cells, followed by their aromatization by aromatase expressed in human granulosa cells. This two-cell system of E₂ biosynthesis in the ovarian follicle is mimicked by theca lutein and granulosa lutein cells of the CL (1).

Estradiol has luteolytic and luteotrophic functions in different species, acting through genomic or nongenomic pathways (2, 3). The expression of E₂ receptors (ER α and ER β) has been described in human and monkey CL (4–6). In addition, 17 β -hydroxysteroid dehydrogenase type 1 acts as a reductase converting estrone (E₁) into E₂ in ovarian cells, and it reaches its maximum expression just before the late luteal phase. Thus, E₂ may act as a paracrine regulator of luteal function and CL life span (7). Interestingly, an alternative form of E₂ regulation of CL function may involve nongenomic pathways. Previous studies from our laboratory have suggested that E₂ modulates P production in human granulosa luteal cells through the inhibition of 3 β -hydroxysteroid dehydrogenase activity (8). These intracrine actions suggest that E₂ regulates human luteal cells function through multiple pathways. On the other hand, the regression of the human CL includes functional and structural changes, including a significant reduction in P secretion and loss of the glandular vascular network (9, 10). Conversely, in the clinical setting the administration of 10,000 IU hCG to women during the mid-luteal and late luteal phases results in a significant increase in plasma P secreted by the CL, and an important reduction of biochemical markers of cell death (11).

There has been increasing interest in the study of estrogens metabolites (EMs), and more specifically E₂ metabolite signaling pathways, mostly in estrogen-producing tissues (12). Several of these compounds have proapoptotic and anti-angiogenic activities (13, 14). Some of these compounds have been tested as anticancer drugs (15). A recent publication from our laboratory indicated that 2-methoxyestradiol (2-ME₂) in physiologic doses in vitro inhibits vascular endothelial growth factor (VEGF) secretion by luteal cells. Interestingly, 2-ME₂ did not prevent hCG stimulation of P biosynthesis by cultured luteinizing granulosa cells (LGCs) (16). In addition, it is known that the human CL produces other EMs, such as other catecholestrogens and keto-estrogens, whose biological activity has not been studied in human ovarian cells.

On the basis of our previous findings and the limited understanding of the role of EMs in human luteal function, the present study was undertaken to [1] assess E₂, E₁, and P and the E₂ metabolites 2-ME₂, 2-methoxyestrone (2-ME₁), 4-hydroxyestrone (4-OHE₁), and 16-ketoestradiol (16-ketoE₂) in luteal tissue throughout the luteal phase, and after hCG administration during the late luteal phase; and [2] examine the effect of EMs on VEGF secretion and angiogenic activity of cultured LGCs in the presence and absence of hCG.

MATERIALS AND METHODS

Clinical and Surgical Trials

Corpus luteum was collected by minilaparotomy from women aged 30–33 years (n = 20) who requested surgical sterilization

at our institution. The participants were healthy, with normal body mass index and regular menstrual cycles, and they had not received any hormonal treatment for at least 3 months before participating in the study. The surgical procedure was scheduled at varying times during the luteal phase and conducted as previously reported (17). The presumptive day of ovulation was determined according to the menstrual pattern, serial vaginal ultrasound, and urinary LH tests. Daily visits of the participant to our outpatient clinic started when the leading follicle reach 14 mm. The day of ovulation was estimated 24 hours after the LH peak. Histologic criteria were also used to confirm the age of the CL. The CL was classified as early CL, 3 to 4 days after LH peak, n = 5; mid-luteal phase CL, 5 to 9 days after the LH peak, n = 5; and late luteal phase CL, 10 to 12 days after the LH peak, n = 5. Late luteal phase CL were stimulated with 10,000 IU of hCG 48 hours before surgery, n = 5.

The institutional review board of San Borja-Arriaran Clinical Hospital approved the study, and written informed consent was obtained from all women participating in the study.

Laboratory Techniques

Tissue for histology and immunodetection was fixed in 4% buffered paraformaldehyde and embedded in paraffin wax. Other pieces of tissue were used for extraction of steroids, or snap-frozen in liquid nitrogen and stored at –70°C for protein determination as described elsewhere (10).

LGC Cultures

Human LGCs were obtained from follicular aspirates of healthy women participating in our IVF program because of male factor infertility (n = 12). The cells were obtained after centrifugation of follicular fluid at 400 × g for 5 minutes. The pellet was suspended, and red blood cells and detritus were removed using a Histopaque (Sigma-Aldrich) gradient. Macrophages were eliminated by preplating for 30 minutes at 37°C, and cultures were completed as previously described (18). The purity of LGCs in culture was assessed by immunodetection of the steroidogenic acute regulatory protein (StAR). This protein it is expressed in GCs after the LH peak in normal cycles or after hCG administration in ovarian stimulation cycles (Supplemental Fig. 1).

Measurement of VEGF

Vascular endothelial growth factor was quantified in conditioned medium (CM) of LGCs cultured for 24 hours under basal conditions and in the absence and presence of hCG (10 IU/mL) with 16-ketoE₂ (0.05 μ M; Sigma-Aldrich), 2-ME₁ (0.05 μ M; Sigma-Aldrich), and 4-OHE₁ (0.05 μ M; Sigma-Aldrich). Conditioned medium was stored at –20°C. Vascular endothelial growth factor was measured by ELISA according to the protocol provided by R&D Systems. The assay includes monoclonal antibody, and samples were assayed in duplicate. The VEGF concentration was measured at 450 nm of absorbance. The intra-assay coefficient of

variation was 2.4%–10.2%; the interassay coefficient of variation was 5.1%–8.7%.

Analysis of EMs

Corpus luteum tissue (100 mg) was homogenized in 0.5 mL of GPB (0.1% gelatin–0.01 M phosphate-buffered saline [pH 7.0]) using a Potter-Elvehjem homogenizer with a tight-fitting Teflon pestle. Steroid extraction was performed with 3 mL of ethyl acetate p.a. (Merck) containing 1% (vol/vol) acetic acid with vortexing for 3 minutes. Tubes were centrifuged (1,100 × *g*) for 10 minutes. The pellet was frozen on dry ice, and the ethyl acetate fraction was transferred to a clean glass tube and evaporated to dryness under nitrogen as previously reported (19). This procedure was performed twice.

The samples were quantified by high performance liquid chromatography (HPLC) equipped with a diode array ultraviolet detector (Agilent), mass spectrometer detector model ZQ MSD (Waters). Twenty microliters of sample was injected to an auto sampler injector (LEAP Technologies) (19).

In Vitro Analysis of Angiogenic Activity

Angiogenic activity was determined in CM obtained from granulosa–lutein cells cultured in the absence and presence of hCG (10 UI/mL) and with and without 16-keto-E₂ (0.05 μM), 2-ME₁ (0.05 μM), and 4-OHE₁ (0.05 μM) for 24 hours. EA.hy926 cells have maintained the phenotype of endothelial cells and were obtained by hybridization of human umbilical vein endothelial cells with the A549/8 human lung carcinoma cell line. The establishment of the EA.hy926 endothelial cell line was reported by, and cells kindly donated by, Dr. Cora-Jean S. Edgell of the University of North Carolina. Cells were collected after trypsin treatment, and 40,000 cells were plated onto matrigel, in the presence of 500 μL of the different media under investigation. EA.hy926 cells were periodically photographed and analyzed using an inverted phase microscope as previously reported (20). Ten representative images per well were recorded and transferred to a computer for image analysis for quantification of an in vitro angiogenic score (AS):

$$AS = \left[\frac{(N^{\circ} \text{ of sprouting cells}) 1 + (N^{\circ} \text{ of connected cells}) 2 + (\text{Number of polygons}) 3}{\text{Total number of cells}} \right] + [0, 1 \text{ or } 2]$$

Western Blotting

Gel electrophoresis was performed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare) as previously reported (16). After transfer, blots were incubated with either anti-catechol-O-methyl transferase (COMT) primary antibody (Sigma–Aldrich) diluted 1:500, or anti-cytochrome P450 1A1 (Millipore) diluted 1:3,000, or anti-β-actin (Sigma–Aldrich) at 4°C overnight. Horseradish peroxidase–conjugated

secondary antibody was used, and chemiluminescence was detected using an Ultra Quant 6.2 Image Reader and analyzed by Image Quant 5.2 software (Molecular Dynamic). Densitometric analysis of immunoreactive bands was normalized to β-actin.

Immunohistochemistry

A Histostain-SP Broad Spectrum kit (Invitrogen) was used for immunostaining. The blocking of peroxidase activity was carried out with 3% H₂O₂ in methanol after antigen retrieval. Nonspecific binding was blocked, and washes between each step were made with phosphate-buffered saline. Sections with anti-VEGF (ab-4) rabbit pAb (Calbiochem) were incubated overnight at 4°C and conjugated with secondary biotinylated antibody and streptavidin–horseradish peroxidase. To visualize bound antibodies, aminoethylcarbazole was used. Images were captured with a light microscope (Olympus BX-51, Olympus Optical) equipped with a CoolSNAP-pro image capture system (Media Cybernetics).

Statistical Analysis

Data are presented as means ± SEM. One-way analysis of variance was performed, followed by a Newman-Keuls test. GraphPad Prism (GraphPad Software) was used to analyze data. Significance was defined as *P* < .05.

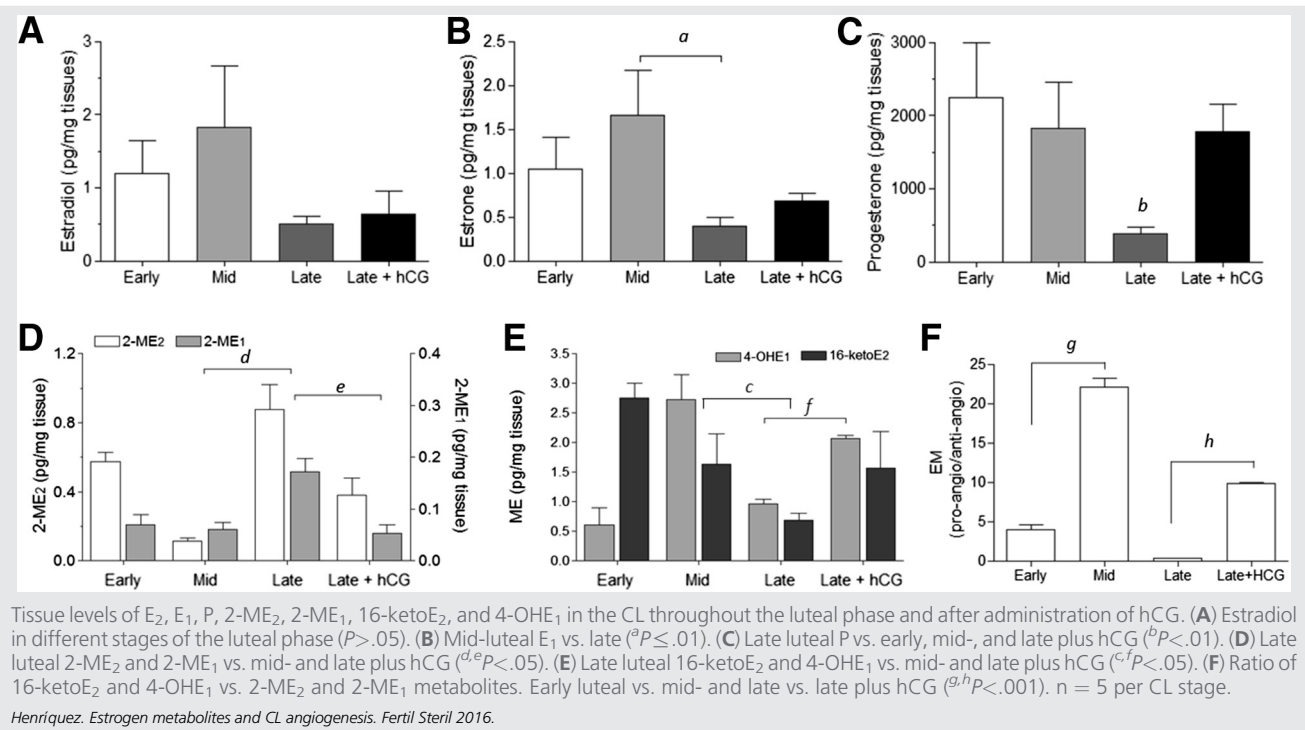
RESULTS

Luteal Tissue Concentration of Steroid Hormones and EMs

Figure 1 shows the tissue concentrations of E₁, E₂, and P in the CL throughout the luteal phase and after administration of a single IM dose of 10,000 IU of hCG. The levels of estrogens and P show similar trends during the study period. The highest luteal tissue concentrations of E₁ and E₂ were observed in mid-luteal phase CL. However, values were significantly different only for E₁ compared with the late

luteal phase (1.59 ± 0.4 vs. 0.5 ± 0.15, *P* ≤ .01). Human chorionic gonadotropin did not increase significantly the tissue levels of E₁ and E₂ as compared with late CL (*n* = 5) (Fig. 1A and B). Exogenous hCG increased significantly the concentration of P in regressing CL (Fig. 1C). 2-Methoxyestradiol was the most abundant methoxyestrogen in the luteal tissue. The lowest levels of 2-ME₂ and 2-ME₁ were detected in mid-luteal phase CL (*P* < .05), followed by a significant increase in late luteal phase CL. Interestingly, hCG administration significantly reduced 2-ME₂ and

FIGURE 1



2-ME₁ tissue levels in regressing CL compared with the late luteal phase ($P < .05$) (Fig. 1D). Conversely, the highest tissue levels of 4-OHE₁ were detected in mid-luteal phase CL. On the other hand, the highest 16-ketoE₂ tissue concentrations were observed in early luteal phase CL ($P < .05$). Both EM tissue levels decreased significantly in the late luteal phase CL compared with the early or mid-luteal phase ($P < .05$). Administration of hCG in doses of 10,000 IU IM restored the tissue concentration of both EMs in the late luteal phase tissue. Figure 1F shows that the ratio of 4-OHE₁ and 16-ketoE₂ vs. 2-ME₂ and 2-ME₁ metabolites is significantly higher in the mid-luteal phase, and after in vivo administration of hCG as compared with late luteal phase CL. The 4-OHE₁ and 16-ketoE₂ metabolites decreased significantly in the late luteal phase CL, but hCG increased the tissue concentrations of these metabolites ($P < .001$). These data suggest a functional role for catecholestrogen and methoxyestrogens in CL development and regression, respectively.

Effect of EMs on LGCs VEGF Production and Angiogenic Activity

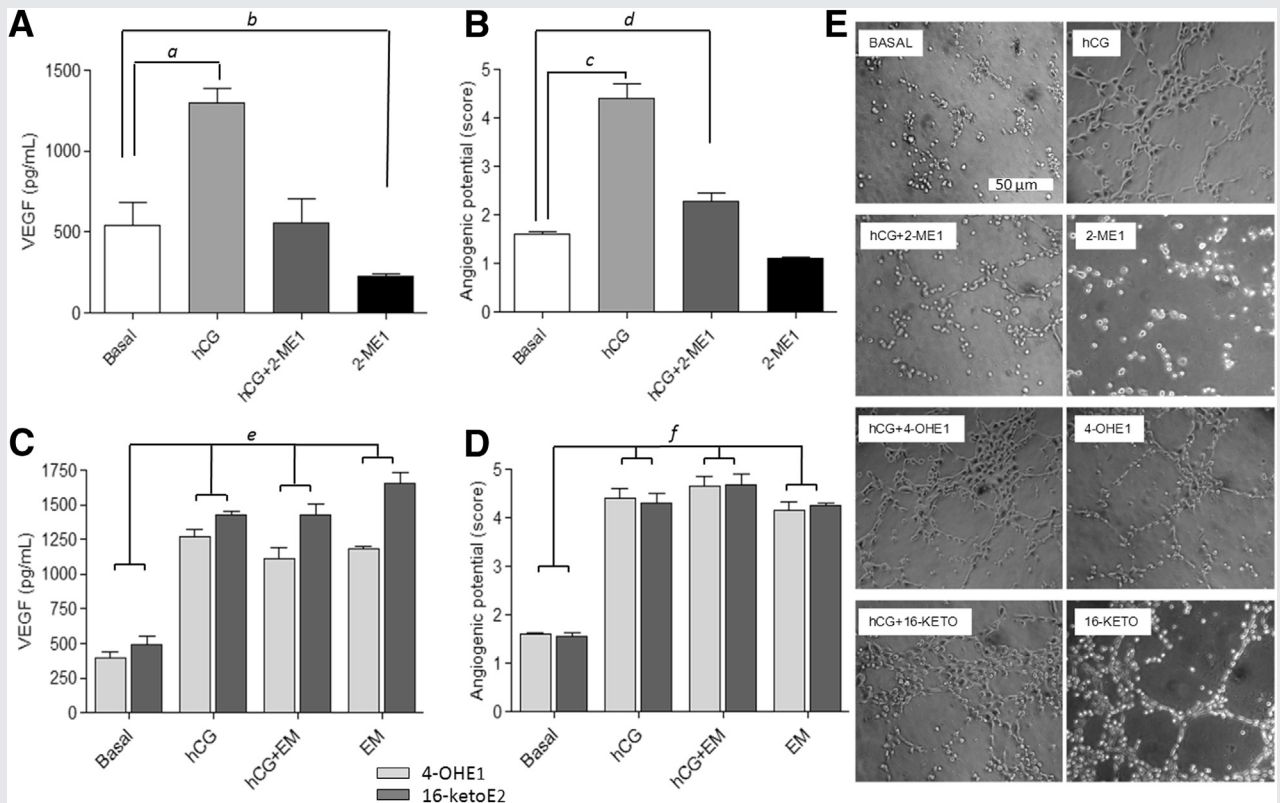
Concentrations of VEGF in CM of LGC cultures increased significantly in the presence of hCG (10 IU/mL) and declined in the presence of 2-ME₁ compared with basal levels ($P < .05$). 2-Methoxyestrogen decreased VEGF levels in the presence of hCG, but this reduction was not statistically significant (Fig. 2A). Figure 2B illustrates the angiogenic activity of LGC cultures. Human chorionic gonadotropin significantly

augments the angiogenic activity of the CM ($P < .05$). 2-Methoxyestrogen treatment significantly reduced tube formation ($P < .05$). Interestingly, hCG prevents the reduction in tube formation. These data suggest that 2-ME₁ does not affect hCG-induced angiogenesis. Figure 2C shows the increasing effect of 4-OHE₁ and 16-ketoE₂ on VEGF secretion compared with basal levels. Both metabolites increased significantly the tube formation ($P < .05$), the proangiogenic effects 4-OHE₁ and 16-ketoE₂ are similar, and their combination did not have a synergistic proangiogenic affect alone or in the presence of hCG (Fig. 2D). These findings suggest an important role for these metabolites in the formation and development of the CL.

Figure 3 illustrates the expression of enzymes involved in methoxyestrogen formation, P4501A1 and COMT, throughout the luteal phase and after administration of hCG. Protein expression of P4501A1 and COMT, determined by Western blotting, did not differ among CL of different ages. However, the abundance of P4501A1 and COMT increased significantly after IM administration of 10,000 IU hCG as compared with late luteal phase CL ($P < .05$). This indicates that hCG regulates enzymes involved in the production of EMs.

Figure 4A depicts the localization of VEGF in CL of different ages and after administration of hCG. The staining for VEGF was predominantly localized in granulosa cells of the CL. The staining in theca-lutein cells of the CL is almost negligible. Figure 4B depicts the VEGF levels evaluated by expression level score (ELS) in LGCs and endothelial cells of early, mid-, and late luteal phase CL and late CL plus hCG compared with early and late luteal phase CL ($P < .05$).

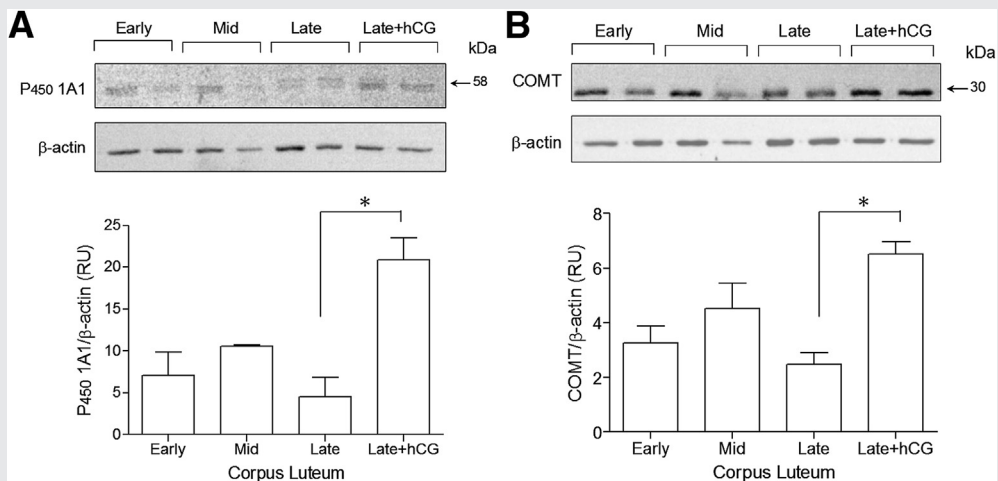
FIGURE 2



Effect of EMs on VEGF production and angiogenic activity by LGC cultures. (A) 2-Methoxyestrone reduced VEGF production by LGCs compared with basal conditions ($^bP < .05$), whereas hCG increased VEGF levels ($^aP < .05$). (B) Human chorionic gonadotropin increased the angiogenic activity ($^cP < .05$), whereas 2-ME1 partially reduced tube forming induced by hCG ($^dP > .05$). (C) 16-Ketoestradiol and 4-OHE₁ increased VEGF production by LGCs compared with basal conditions ($^eP < .05$). (D) Both EMs increased tube formation compared with basal conditions ($^fP < .05$). (E) Photomicrograph of the angiogenic assay.

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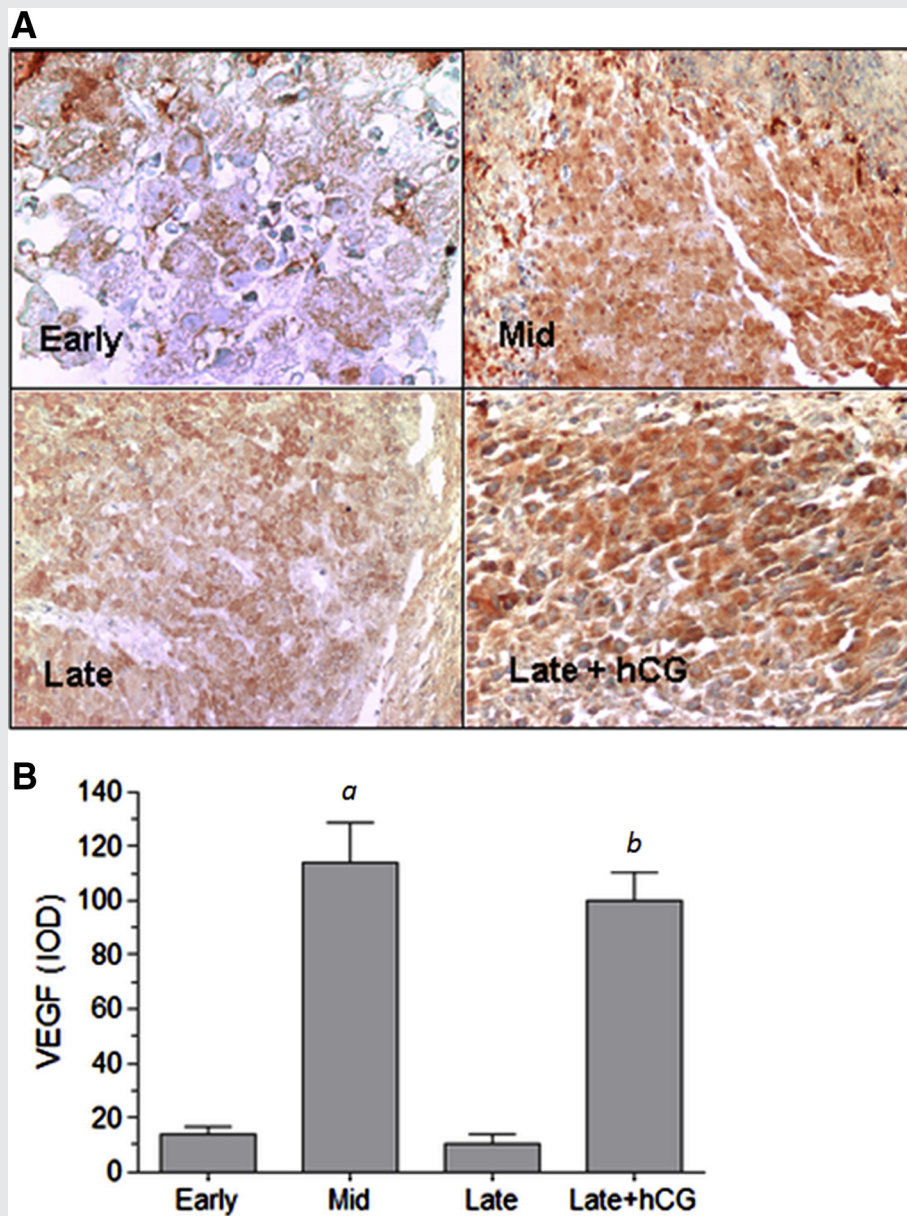
FIGURE 3



Expression of COMT and P4501A1 in CL collected during the luteal phase and after administration of hCG. (A, B) Western blotting for P4501A1 and COMT. Protein expression of P4501A1 and COMT did not differ between CL of different ages. In vivo hCG administration enhanced expression ($^*P < .05$, $n = 5$).

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FIGURE 4



Immunohistochemistry of VEGF throughout the luteal phase and after administration of hCG. (A) Photomicrographs depict luteal tissue collected during the early, mid-, late, and late plus hCG luteal phase stained for VEGF. (B) The immunohistochemistry signal is prominent in luteal cells of the mid-luteal phase CL and after hCG administration compared with the early and late luteal phase CL (^{a,b} $P < .05$, $n = 5$).

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DISCUSSION

The human CL is an endocrine gland characterized by significant angiogenic and steroidogenic activity in both the normal menstrual cycle and during early pregnancy (1). The molecular and cellular machinery controlling angiogenesis and steroidogenesis is largely regulated by LH or hCG (21, 22). It is thought that VEGF, connective tissue growth factor, and the insulin growth factor (IGF) systems are also involved in the regulation of luteal function and angiogenesis (23–25). Interestingly, very recently we

reported that 2-ME₂ produced by luteal cells may play a role in physiologic luteolysis by reducing luteal angiogenesis (16). However, 2-ME₂ is not the only EM produced by the CL that may have biological functions that are not associated with their parent hormones, E₂ and E₁. Because of the limited understanding of the role of EM in human luteal function, the aim of the present study was to determine whether other EMs produced by the human CL are involved in luteal angiogenesis and function. Previous reports suggest that swine granulosa cells cultured under restricted oxygen conditions produce

increased amounts of 2-ME₂, which may explain the reduction in VEGF in vitro. The mechanism presumably involves the regulation of hypoxia-inducible factor 1 α expression. Interestingly, this inhibitory effect was reversed by hCG (26, 27). It is tempting to speculate that the increasing expression of hypoxia-inducible factor 1 α in cells exposed to hypoxia fails to activate VEGF transcription in the presence of methoxyestrogens. This physiologic setting of hypoxia could be present during CL regression. Consequently, we analyzed a spectrum of these molecules, including keto, hydroxylated, and methylated EMs, in CL tissue of different ages. The selection of the EMs potentially involved in luteal function was based on their concentrations in luteal tissue of different ages and on reports in the scientific literature (28–30).

The second aim of this investigation was to examine in vivo and in vitro the effect of EMs and hCG on the steroidogenic competence and angiogenic potential of granulosa-lutein cells. It is well known that the early and mid-luteal phase human CL is a site of a rich vascular network essential for P biosynthesis (21). It is thought that LH and VEGF play a critical role in luteal vasculature development (31). Our data indicate that the luteal levels of 16-ketoE₂ were greater in early luteal phase CL. Thus, it is conceivable that 16-ketoE₂ may play a significant role in the vascular development of early luteal phase CL because it increases tube formation activity of EA.hy 926 cells. This proangiogenic effect of 16-ketoE₂ could partially be explained by our experimental data that demonstrate an increase of VEGF synthesis. The lack of a synergistic effect between hCG and 16-ketoE₂ on angiogenic activity may suggest that both compounds use the same signaling pathways. Mid-luteal phase tissue showed a significant increase in 4-OHE₁ as compared with early luteal phase CL. Interestingly, both 16-ketoE₂ and 4-OHE₁ have similar effects on VEGF secretion and angiogenic activity.

In our initial study we discovered that 2-ME₂ was a prominent EM in late luteal phase tissue, potentially playing an important role in physiologic luteal regression. In the present study we found that other members of the methoxyestrogens family, including 2-ME₁, are significantly elevated in late luteal phase tissue in association with a significant reduction of VEGF secretion and angiogenic activity. Similar effects of both methoxyestrogens and concentration changes in late luteal CL suggest their participation in CL regression during nonconception cycles. Taken together, these new findings on EMs indicate that they may be important players in ovarian physiology. The ratios of EMs with proangiogenic and antiangiogenic action were determined throughout the normal luteal phase suggest a finely tuned paracrine effect at the time of implantation and CL regression. An additional important finding was derived from the in vivo study of the acute effects of hCG, which caused a significant reduction of 2-ME₂ and 2-ME₁ in late luteal phase tissue, with a parallel increase in VEGF and the pro-angiogenic EMs 16-ketoE₂ and 4-OHE₁.

P4501A1 and COMT are critical enzymes in EM production (32). In our previous study the abundance of these enzymes determined by Western blot did not change significantly in the CL throughout the luteal phase (16). In

contrast, in the present study, the in vivo IM administration of 10,000 IU of hCG significantly increased the expression of P4501A1 and COMT enzymes 24 hours after hCG administration. The increase in P4501A1 abundance may support the rise of the proangiogenic EMs like 16-ketoE₂ and 4-OHE₁ that presumably contribute to support the CL vascular network (30). However, the increase in COMT abundance promoted by hCG is not consistent with the reduction of 2-ME₂ and 2ME₁ concentrations in the late luteal phase CL. This apparent discrepancy may reflect the role of COMT in other metabolic activities, or perhaps alterations in other enzymes involved in EM processing that would divert substrate away from COMT, such as enzymes responsible for estrogen sulfoconjugation and glucuronidation, consequently reducing the levels of catecholestrogens (33, 34).

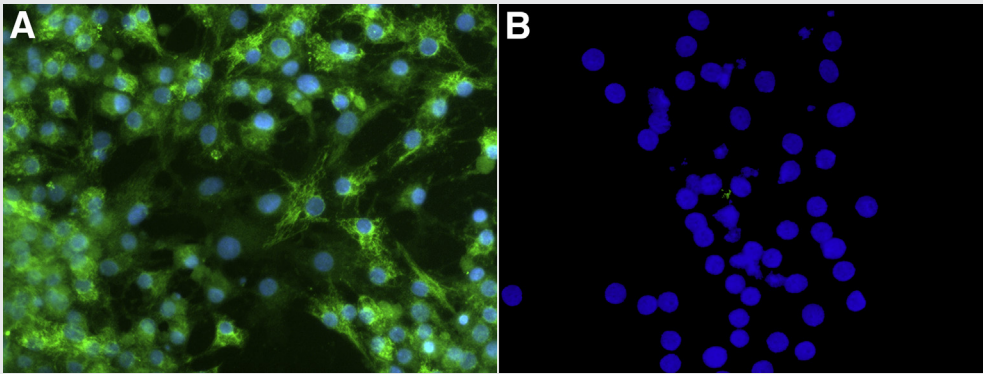
In summary, our observations suggest novel paracrine mechanisms, driven by different EMs, in the development and demise of the human CL during nonconception menstrual cycles, and the late luteal phase rescue of the CL by hCG in cycles of conception.

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SUPPLEMENTAL FIGURE 1



Immunodetection of StAR in cultured LGCs. **(A)** Immunofluorescence staining for StAR in primary culture of LGCs under basal conditions. The StAR signal (*green*) is intense in the cytoplasm (mitochondria) of most cultured cells. **(B)** Negative control (absence of primary antibody).

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