ORIGINAL ARTICLES: REPRODUCTIVE ENDOCRINOLOGY

2-Methoxyestradiol in the human corpus luteum throughout the luteal phase and its influence on lutein cell steroidogenesis and angiogenic activity

Paulina Kohen, B.Sc.,[a] Soledad Henríquez, Ph.D.,[a] Candy Rojas, B.Sc.,[a] Phillip M. Gerk, Ph.D.,[d] Wilder A. Palomino, M.D.,[a,b] Jerome F. Strauss III, M.D., Ph.D.,[c] and Luigi Devoto, M.D.[a,b]

[a] Institute for Maternal and Child Research (IDIMI) and [b] Department of Obstetrics and Gynecology, Faculty of Medicine, San Borja-Arriaran Clinical Hospital, University of Chile, Santiago, Chile; and [c] Department of Obstetrics and Gynecology, School of Medicine, and [d] School of Pharmacy, Virginia Commonwealth University, Richmond, Virginia

Objective: To quantitate 2-methoxyestradiol (2-ME) in human corpus luteum (CL) of different ages and to determine the expression of cytochrome-P450-1A1 (CYP1A1) and catechol-O-methyl transferase (COMT) in CL and the action of 2-ME on P, vascular endothelial growth factor (VEGF) secretion, and luteal angiogenesis.
Design: Experimental study.
Setting: University division of reproductive endocrinology.
Patient(s): Twenty-four women of reproductive age.
Intervention(s): CL was collected from 15 women during the minilaparotomy for tubal sterilization. Granulosa lutein cells were harvested 36 hours after hCG administration in patients undergoing IVF.
Main Outcomes Measure(s): Levels of 2-ME were determined by high-performance liquid chromatography in CL. CYP1A1 and COMT were assessed by immunohistochemistry and Western blot. P and VEGF were measured by radioimmunoassay and ELISA. The angiogenic potential was analyzed using EA.hy926 cells.
Result(s): Plasma levels of E_2 decreased in the late luteal phase in association with an increase in luteal tissue of 2-ME concentrations. Concomitantly, there was a significant reduction of angiogenic activity in late CL. There was no significant variation in CYP1A1 and COMT expression in all CL. In physiological doses, 2-ME inhibited basal VEGF by granulosa lutein cells and diminished the angiogenic activity in conditioned media but did not prevent P and VEGF production stimulated by hCG.
Conclusion(s): These data suggest the participation of 2-ME in physiological luteolysis by reducing angiogenesis. However, 2-ME did not prevent in vitro hCG stimulation of P biosynthesis, providing a mechanism for CL rescue in the cycle of conception. (Fertil Steril® 2013;100:1397–404. ©2013 by American Society for Reproductive Medicine.)
Key Words: 2-methoxyestradiol, human corpus luteum, angiogenic potential, vascular endothelial growth factor

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The cyclic secretion of E_2 throughout the reproductive process in women depends on repetitive follicle recruitment and single dominant follicle selection, followed by the LH surge that terminates the program of FSH-dependent steroidogenesis and granulosa cell growth. Concomitantly, LH induces the expression of genes required for ovulation,

while promoting differentiation of granulosa cells (GCs) into luteal cells. E_2 biosynthesis during the human follicular phase as well during the luteal phase is dependent on the production of androgens production by theca cells, while CYP1A1 (P450arom) expressed in CGs catalyzes E_2 synthesis from the thecal androgens. The model of estrogen biosynthesis, characteristic of the ovarian follicle, is mimicked by theca and granulosa lutein cells of the human corpus luteum (CL). Interestingly; CYP1A1 expression during the follicular phase is FSH dependent, while in the luteal phase it is LH dependent [1].

It is important to highlight that the primate CL, in contrast to that of rodents, retains the ability to produce high levels of E_2 (150–200 pg/mL), particularly during the midluteal phase. However, E_2 secretion by the human CL does not appear to be as critical for pregnancy as is P, since its replacement does not sustain pregnancy. The physiological role of luteal E_2 is unknown, although it has been postulated to be involved in luteolysis in primates, where the luteolytic process is independent of uterine prostaglandins. Previous studies from our laboratory suggested that E_2 modulates P production in human GC cultures by the reduction of 3β-hydroxysteroid dehydrogenase activity [2].

In the normal menstrual cycle, the process of luteolysis encompasses a loss of functional and structural integrity of the CL. It is thought that luteolysis is determined by factors downstream from the LH receptors [3]. Recently, there has been interest in the E_2 metabolic pathways, mostly in estrogen-producing tissues. There are several metabolic fates, including sulfonation, glucoronidation, hydroxylation, and methylation [4]. The 2-Methoxyestradiol (2-ME) has a significant negative impact on angiogenesis and cell proliferation [5–8]. Based in those findings, several promising preclinical oncology trials have been conducted [9, 10].

The human CL produces a number of E_2 metabolites. However, the physiological functions of these metabolites are poorly understood. Consequently, the aims of this study were [1] to determine the tissue concentrations of 2-ME in the CL of different ages; [2] to establish the localization and expression of the enzymes involved in 2-ME synthesis, cytochrome CYP1A1 and cathecol-O-methyl transferase (COMT), in the CL of different ages; [3] to study the effect of 2-ME on P production by granulosa lutein cells; and [4] to examine the effect of 2-ME on vascular endothelial growth factor (VEGF) secretion, production, and angiogenesis.

MATERIALS AND METHODS

The study was approved by the Hospital Clínico San Borja-Arriarán Medical Ethics Committee, and signed informed consent was obtained from all women participating in this study. The CL was collected from women (n = 15) at the time of mini-laparotomy for tubal sterilization. The surgery was scheduled at varying times throughout the luteal phase as described elsewhere [11]. All women were healthy, aged 33–40 years, with normal body mass index and regular menstrual cycles, and had not received any form of hormone treatment for at least

3 months before participating in the study. Blood was collected before surgery for P and E_2 determinations.

The presumptive day of ovulation was determined by serial vaginal ultrasound and urine LH samples. The CL was classified as early, mid, and late using biochemical and histological parameters as defined elsewhere [11].

The CL was enucleated from the ovary and transported to the laboratory under sterile conditions. Tissue for histology and immunodetection was fixed in 4% buffered paraformaldehyde and embedded in paraffin wax. Other pieces were used for extraction of steroids or culture of luteal cells or snap-frozen in liquid nitrogen and stored at $-70°C$ for protein extractions.

Luteal Cell Dispersion and Culture

Luteal cells of midluteal phase CL were dispersed as described elsewhere [11]. Briefly, CL were enzymatically dissociated in M-199 containing $NaHCO_3$ (26 nmol/L), penicillin (50 IU/mL), bovine serum albumin (BSA; 0.1% w/v), collagenase (370 IU/ 100 mg tissue), and DNase (14 Kunitz units/100 mg tissue). The cell viability was 85% and assessed by the trypan blue exclusion method. Total luteal cells (0.8×10^5 live cells/well) were cultured in serum-free medium in the absence and presence of hCG (10 IU/mL) and 2-ME (0.05 μM; Sigma) for 24 hours. Culture medium was collected and stored at $-20°C$ until assayed for P determinations by radioimmunoassay and VEGF by ELISA.

Granulosa Lutein Cell Culture

Human GCs were isolated from follicular aspirates of women undergoing IVF after standard follicular hyperstimulation due to male factor infertility. Briefly, the CGs were obtained by centrifugation at 400 g for 5 minutes. The pellet was suspended in phosphate-buffered saline (PBS) EDTA 1 mM and washed. The red blood cells and detritus were eliminated in Histopaque gradient. Macrophages were eliminated by additional plating of the cells for 30 minutes at 37°C. The GCs were cultured for 76 hours in growth medium M-199 with 10% heat-treated fetal calf serum and 24 hours in serum-free M-199 supplemented with 0.1 % BSA [12]. Cultures were treated for 24 hours with different compounds.

Western Blotting

Equal amounts of CL protein extracts were separated by 10% SDS-PAGE and transferred to PVDF membranes (GE Healthcare). The blots were blocked in 5% fat-free milk in T-TBS (20 mM Tris, 500 mM NaCl, 0.01% Tween 20) for 1 hour and probed with primary antibodies COMT (Sigma-Aldrich), CYP1A1 (Millipore), and β-actin (Sigma-Aldrich) overnight at 4°C. Immunoreactive products were detected using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection system (ECL Western blotting reagents, GE Healthcare) and visualized using an Ultra Quant 6.2 Image Reader. Membranes were tested with anti-β-actin antibody as a loading control.

Densitometric analysis of specific immunoreactive bands was performed using ImageQuant 5.2 software (Molecular Dynamics Inc.).

FIGURE 1
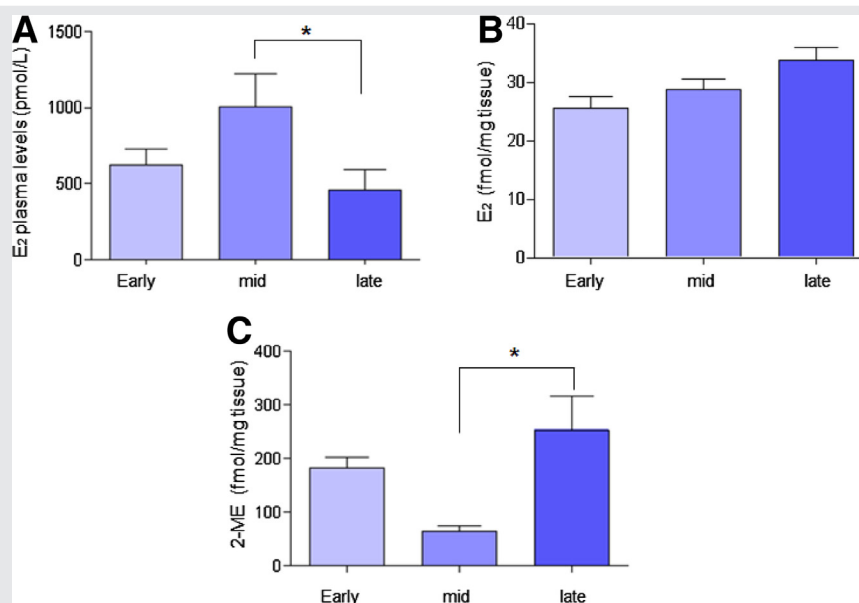
Levels of 2-ME and E_2 in luteal tissue and plasma. (A) Plasma levels of E_2 decreased in the late luteal phase ($P<.05$) compared with in the midluteal phase. (B) E_2 tissue concentrations in different stages of the luteal phase. (C) Tissue concentrations of 2-ME are increased in the late luteal phase CL compared with in the midluteal phase CL (n = 9, $P<.05$).

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Immunohistochemistry

Immunostaining was carried out according to an established protocol using the Histostain-SP Broad Spectrum kit (Invitrogen Corp.). After antigen retrieval, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. Nonspecific binding was blocked, and washes between each step were carried out in PBS. Sections were incubated overnight at 4°C with anti-COMT rabbit polyclonal antibody (Sigma-Aldrich), anti-cytochrome CYP1A1 polyclonal (Millipore), anti-VEGF polyclonal (Calbiochem), and anti-VEGF-R2 (Flk-1/KDR) polyclonal (Abcam, Inc.), followed by incubation with a biotinylated secondary antibody for 30 minutes and streptavidin-horseradish peroxidase. Bound antibodies were visualized with 3,3′-diaminobenzidine tetra-hydrochloride. Images were captured with a light microscope Olympus BX-51TF (Olympus Optical Co. Ltd.) and Cool-Snap-pro-media-Cybernetics.

Measurement of VEGF

VEGF was determined in conditioned medium (CM) collected from early, mid, and late luteal phase CL cells cultured for 24 hours in basal conditions and GCs cultured in the absence and presence of hCG (10 IU/mL) and 2-ME (0.05 μM) for 24 hours. Culture medium was collected and stored at −20°C. VEGF was determined by ELISA following the manufacturer's protocol provided by R&D Systems, Inc. The assay used monoclonal antibody, samples were assayed in duplicate, and the concentration of VEGF was determined for absorbance at 450 nm. The intra-assay coefficient of variation was 2.4%–10.2%; the interassay coefficient of variation was 5.1%–8.7%.

Analysis of 2-ME

Tissue from individual CL (100 mg) was homogenized in 0.5 mL of gelatin phosphate buffer saline (0.1% gelatin-100 mM PBS, pH 7.0) with a Potter-Elvehjem homogenizer with Teflon pestle. Samples were extracted in glass extraction tubes with 3 mL of ethyl acetate p.a. (Merck) containing 1% acetic acid and vortexed for 3 minutes and then centrifuged at 1,100 g for 10 minutes. The pellet was frozen in dry ice, and the ethyl acetate fraction was transferred to another glass tube and evaporated to dryness under nitrogen. This procedure was done twice, and the ethyl acetate fractions were dried in the same tube.

The 2-ME was analyzed as described elsewhere (13), with modifications. After extraction, the samples were reconstituted in 200 μL of 25% acetonitrile (1% aqueous acetic acid), vortexed, centrifuged, and aliquoted into high-performance liquid chromatography vials. Reconstituted samples (70 μL) were injected using a 2695 autoinjector (Waters) onto an Alltima HP C18 4.6 × 100 mm 3 μm column (Grace Davison). The 2-ME was separated from other phase 1 and phase 2 metabolites of estrone, E_2, and estriol using gradient elution (25% acetonitrile 75% aqueous [0.05% trifluoroacetic acid]) for 9 minutes, then 35% acetonitrile 65% aqueous for 8 minutes, then returning to 25% acetonitrile 75% aqueous for 2 minutes. Detection was performed using a Waters 2475 fluorescence detector with excitation at 288

FIGURE 2
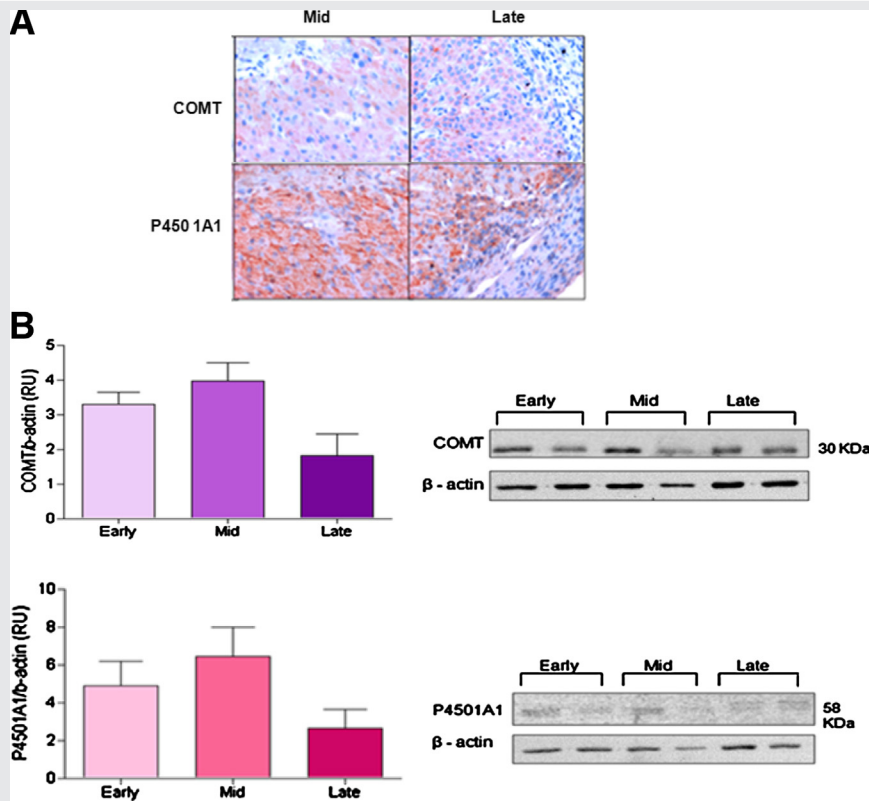
Immunolocalization of COMT and CYP1A1 in CL collected during the luteal phase. (A) Representative photomicrograph of midluteal phase and late luteal phase tissue stained for COMT and CYP1A1. The staining is positive in granulosa lutein cells. (B) Protein expression of COMT and CYP1A1 assessed by Western blotting did not differ between CL of different ages.

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nm and emission monitored at 337 nm. Under these conditions, 2-ME eluted at 16.4 minutes; calibration curves were linear between 140 and 140,000 fmol per 70 μL injection. As a control, the 2-ME concentrations measured in serum from five pregnant women (12–27 weeks gestation; obtained from BioChemed) were 2.9 $\pm$ 1.7 ng/mL, consistent with published values (17).

In Vitro Analysis of the Angiogenic Activity

To determine angiogenic activity, luteal cells were cultured in the absence and presence of hCG (10 IU/mL), 2-ME (0.05 μM), or a mixture of both. After 24 hours of incubation, the medium, now termed CM, was collected and added to EA.hy926 cells (an endothelial cell line) and seeded onto matrigel. In addition, the endothelial cells lines were incubated with hCG, 2-ME, and a mixture of both to test the direct effect of these compounds. The matrigel was polymerized for 1 hour at 37°C with 5% CO_2. EA.hy926 cells were trypsinized, and 40,000 cells were plated onto matrigel in the presence of the CM 500 μL under investigation to assess the pro- or anti-angiogenic activity (14).

The EA.hy926 cells were observed periodically and photographed using an inverted phase contrast microscope with a 20× objective. Ten representative images

per well were recorded and transferred to a computer for image analysis for quantification of an in vitro angiogenic score.

Statistical Analysis

E_2 and 2-ME concentrations were determined in triplicate in luteal tissue of different ages. Data are presented as a mean $\pm$ SEM. Statistical analyses were performed by analysis of variance followed by a Tukey test. Significance was defined as $P < .05$. Data were analyzed using GraphPad Prism (GraphPad Software Inc.).

RESULTS
E$_2$ and 2-ME Concentrations in the CL throughout the Luteal Phase

Figure 1 shows the plasma levels of E_2 throughout the luteal phase and tissue levels of E_2 and 2-ME in the CL of different ages. The lowest plasma levels of E_2 were observed in the late luteal phase ($P < .05$; Fig. 1A). E_2 tissue concentrations did not vary in the CL of different ages (Fig. 1B). The highest luteal tissue concentration of 2-ME was observed in late luteal phase CL ($P < .05$), compared with midluteal phase 2-ME concentrations (Fig. 1C).

FIGURE 3
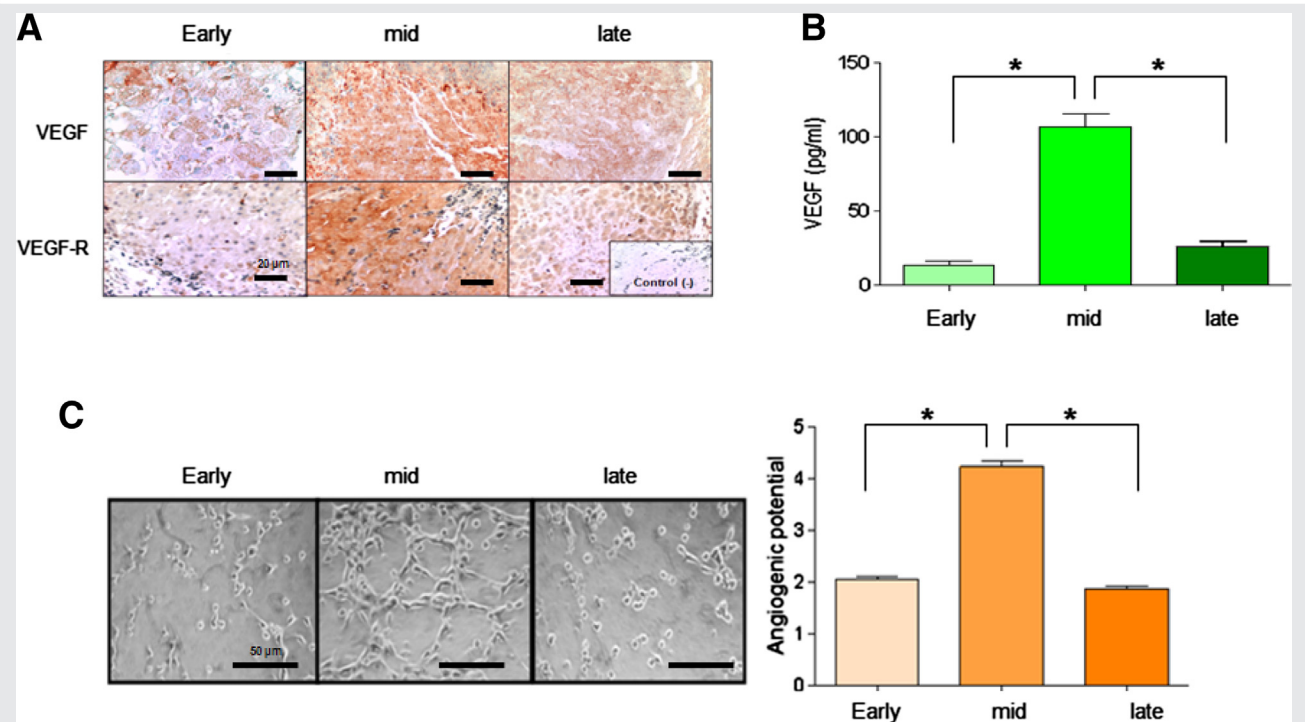
Immunohistochemistry of VEGF and VEGF-R in CL of different ages and angiogenic factor assays. (A) The photomicrograph represents luteal tissue collected during early, mid-, and late luteal phases stained for VEGF and VEGF-R, showing prominent staining in the midluteal phase CL compared with in the early and late luteal phase CL (n = 5, P<.05). (B) VEGF levels determined in culture media of luteal cells is greater in midluteal phase CL cells compared with in early and late luteal phase CL cells (n = 9, P<.05). (C) The photomicrograph represents the angiogenic assay of luteal cell culture of different ages. CM from midluteal phase cell cultures significantly increased the angiogenesis (P<.05).

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Expression of Enzymes Involved in 2-ME Synthesis in the CL

We next analyzed the expression of CYP1A1 and COMT, the key enzymes involved in 2-ME production. Figure 2 illustrates the localization and abundance of CYP1A1 and COMT throughout the luteal phase. Immunohistochemistry primarily localized the enzymes in luteal steroidogenic cells. Protein expression of CYP1A1 and COMT, determined by Western blotting, did not differ among the CL of different ages (P>.05).

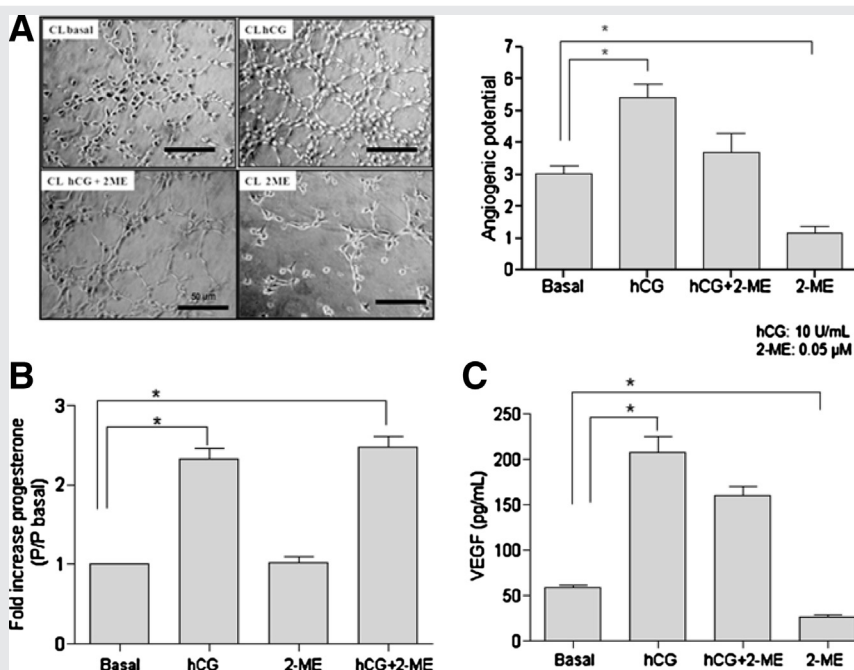### Effects of 2-ME on Angiogenic Activity and Steroidogenesis

Figure 3A shows the localization of VEGF and VEGF receptor (VEGF-R) in the CL of different ages. VEGF and VEGF-R were significantly higher in granulosa luteal cells and endothelial cells of midluteal phase CL compared with early and late luteal phase CL (P<.05) according to the Expression Level Score. This is consistent with the pattern of VEGF production in CM as determined by ELISA from cultured luteal cells of different ages (Fig. 3B). Panel C shows images of the angiogenic activity of CM from luteal cell cultures of different ages. The highest angiogenic activity was found in midluteal phase CL cell cultures (P<.05), and EA.hy926 cells display the

greater capillary-like structures and complex type polygons, reinforced in thickness with two to three or more cells.

CM from midluteal phase cell cultures after 24 hours of incubation with hCG (10 U/mL), 2-ME (0.05 μM), or a mixture of both was incubated with EA.hy926 cells for 12 hours. We observed that hCG significantly increased the angiogenic activity in the luteal cell CM (P<.05), which is reflected in the formation of capillary-like structures and more complex structure polygons, while 2-ME treatment significantly reduced tube formation (P<.05). In addition, 2-ME reduced tube formation induced by hCG, but this reduction did not achieve statistical significance (Fig. 4A). To rule out the direct effect of hCG, 2-ME on the endothelial cells, we treated the endothelial cell cultures with these compounds but found no effect on growth or morphology (Supplemental Fig. 1). Figure 4B illustrates the effect of 2-ME on P production by cultured luteal cells. As expected, hCG significantly increased P production by luteal cells (P<.05), while 2-ME at a physiological dose, determined from the CL concentrations (0.05 μM), did not reduce basal P production or P production stimulated by hCG.

The dose response curve for 2-ME (0.01, 0.05, 0.1, 0.5, and 1 μM) indicated that the concentration of 0.05 μM 2-ME did not affect cell viability (82%–88% compared with controls; data not shown). Doses beyond 1 μM significantly decreased cell viability.

Effect of 2-ME on angiogenic activity and P secretion by cultured luteal cells. (A) Photomicrograph represents the angiogenic assay. The 2-ME significantly reduced basal tube formation (angiogenic activity) secreted by luteal cells, while hCG significantly increased the angiogenic activity ($P<.05$). In addition, 2-ME also reduces tube-forming activity induced by hCG, but this reduction was not statistically significant. (B) Effect of 2-ME on P production by luteal cells cultures. HCG significantly increased P production by luteal cells ($P<.05$), while 2-ME (0.05 μM) did not affect basal P production or hCG-stimulated P secretion. (C) 2-ME significantly reduced VEGF production by granulosa lutein cells, while hCG significantly increased the VEGF levels ($P<.05$). The 2-ME also reduced VEGF secretion induced by hCG, but this reduction was also not statistically significant.

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We also determined the VEGF levels in CM of granulosa lutein cells, cultured with hCG (10U/mL), 2-ME (0.05 μM), or 2-ME plus hCG. The VEGF levels increased significantly in the presence of hCG, while 2-ME significantly reduced levels compared with basal levels ($P<.05$). The 2-ME also decreased VEGF levels induced by hCG, but this reduction was not statistically significant (Fig. 4C).

DISCUSSION

Several studies revealed that 2-ME, a natural metabolite of 17β-E$_2$ devoid of estrogenic activity, is a multitarget steroid that acts in several tissues (15, 16). It is thought that during the normal menstrual cycle, the principal source of 2-ME is the CL (17). However, the physiological significance of its production throughout the human luteal phase is not understood. A number of investigators have described the antiangiogenic, antiapoptotic, and antiproliferative effect of 2-ME in different biological contexts (18–20). These activities are all important to ovarian physiology, as they take place in follicular development, CL formation, and regression (21, 22).

Angiogenesis is an important feature of early and midluteal phase CL, and diminished cell proliferation and progression of the apoptosis program are characteristic features of luteolysis (23). Therefore, the aim of this investigation was to determine

the physiological concentrations of 2-ME within the CL of different ages. Interestingly, the highest concentrations of 2-ME were discovered in late luteal phase tissue. Conversely, the lowest plasma E$_2$ levels were found in the late luteal phase. These findings of sustained E$_2$ levels within the luteal tissue throughout the luteal phase, and the increasing levels of 2-ME in late CL associated with decreasing plasma levels of E$_2$ in late luteal phase, suggest that in the late CL there is an increase in E$_2$ metabolism via the ME pathway at the time of normal luteolysis.

Several studies have demonstrated that CYP1A1 and COMT are critical enzymes in 2-ME production (24). We hypothesized that changes in the expression of these enzymes could explain the cyclic variation of 2-ME in the CL. However, the abundance of CYP1A1 and COMT, determined by Western blotting, did not change significantly in the CL of different ages. These data may suggest that the changes in luteal tissue 2-ME levels are not dependent on the expression of these enzymes and alternative enzymes, particularly those that can estradiol 2-hydroxylation may be responsible for our observations (25). Moreover, we did not determine enzyme activity, and the catalytic function of these enzymes may have changed, resulting in increased synthesis of 2-ME.

P has been considered the key steroid hormone of the CL. There is diminished P biosynthesis by the late luteal phase CL that may be regulated by intraovarian factors. Therefore, we

studied the in vitro effect of 2-ME on P synthesis by luteal cells culture. Our findings indicated that 2-ME in physiological concentrations does not affect basal or hCG-stimulated P production.

The CL is a major site of angiogenesis and neovascularization in the early and midluteal phases (21, 22). However, angiogenesis must be controlled to permit luteal regression in an infertile 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 the CL of different ages. Other investigators have previously reported greater mRNA expression of VEGF-A and VEGF in midluteal phase CL (26, 27). Our data on VEGF secretion by cultured luteal cells are consistent with these observations. We also evaluated the angiogenic activity in the CM from midluteal phase cells using the EA.hy926 endothelial cell line. The 2-ME significantly diminished the angiogenic activity of luteal cells, suggesting a detrimental intraovarian action of 2-ME through the antiangiogenic potential of this E_2 metabolite. This result is consistent with the reduction in VEGF levels observed in granulosa lutein cells cultured with 2-ME. Collectively, these findings suggest that the increased intraluteal 2-ME levels in late luteal phase CL contribute to the reduction of VEGF levels, which may explain the reduction in angiogenic activity at this stage. Interestingly, GC cultures from other species treated with 2-ME in high doses react with decreased VEGF production (7, 8, 28). However, the mechanism through which 2-ME suppresses VEGF and angiogenic activity is unknown. Interestingly, 2-ME does not inhibit the proangiogenic effects of hCG, which would allow rescue of the late luteal phase CL in a cycle of conception. It remains to be determined how hCG overcomes the inhibitory effect of 2-ME. In addition to the 2-ME effects on angiogenic function, it is feasible that the reduction of VEGF production could favor the apoptotic program of luteal cells.

In conclusion, these findings suggest that the alterations in 2-ME concentrations in the late CL could be the result of other enzymatic routes to E_2 hydroxylation, access of precursors to the key enzymes, or an increase in tissue clearance of 2-ME, through glucuronidation or oxidation. Physiological doses of 2-ME do not affect P production and do significantly reduce basal but not hCG-stimulated VEGF and angiogenic factor elaboration. It is tempting to speculate that the intraovarian action of this metabolite could contribute to the functional and structural vascular regression of luteal tissue during normal luteolysis and account for the luteolytic actions of estrogen that have been described in the primate ovary. Further studies on different estrogen metabolites during CL development, function, regression, and rescue by hCG may lead to new physiological and pathophysiological information that could contribute to a better understanding of luteal function and dysfunction.

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SUPPLEMENTAL FIGURE 1
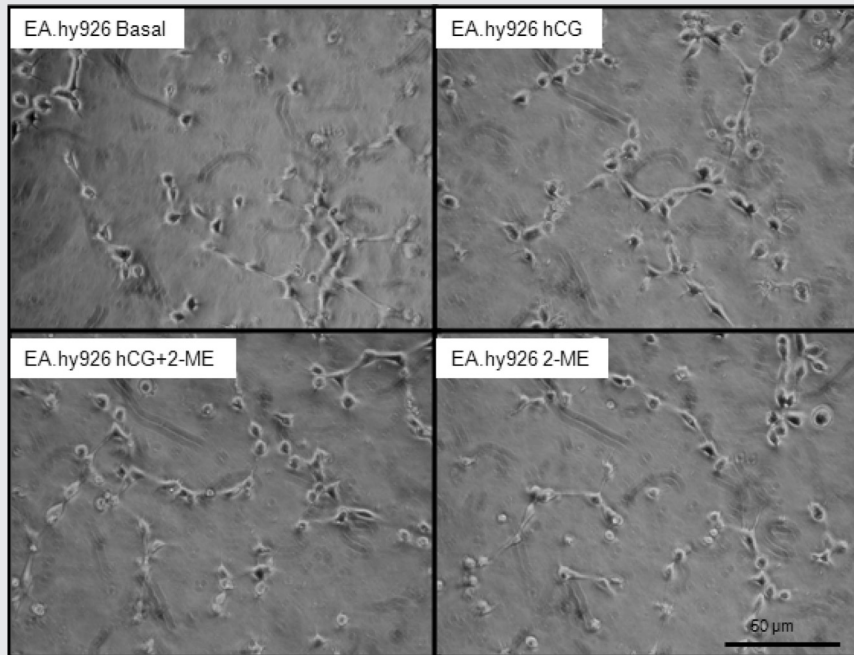
The photomicrograph represents the angiogenic action of 2-ME, hCG, and 2-ME plus hCG on endothelial cells (EA.hy926). These compounds did not affect basal tube formation.

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