Pregnancy Ameliorates the Inhibitory Effects of 2-Methoxyestradiol on Angiogenesis in Primary Sheep Uterine Endothelial Cells

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Abstract

The estrogen metabolite 2-methoxyestradiol (2-ME2) is one of the most potent antiangiogenic and proapoptotic endogenous steroids. Herein, we investigate the effects of 2-ME2 on angiogenesis of cultured primary ovine uterine artery endothelial cells (UAECs) from nonpregnant follicular (F-UAECs), nonpregnant luteal (L-UAECs), and pregnant ewes (P-UAECs). Uterine artery endothelial cells were treated with vehicle control, 10^{-8} mol/L 17 β -estradiol (17 β E2), or 10^{-9} to 10^{-6} mol/L 2-ME2. Angiogenesis, apoptosis, and cell morphology were assessed by capillary tube formation, flowcytometry, and immunohistochemistry. 17 β E2 stimulated while 10^{-6} mol/L 2-ME2 inhibited capillary tube formation in F-UAECs ($P < .05$). The inhibitory effects of 2-ME2 on angiogenesis were minimal in L-UAECs and were absent in P-UAECs when compared to controls. 10^{-6} mol/L 2-ME2 increased apoptosis and inhibited microtubular structure equally in pregnant and nonpregnant UAECs when compared to control or 17 β E2 treatments. Thus, 2-ME2 inhibit capillary tube formation in F-UAECs while L-UAECs and P-UAECs are relatively unresponsive to the inhibitory effects of 2ME2 indicating that the pregnancy phenotypic state of the UAECs may modulate the action of 2-ME2 on capillary angiogenesis.

Keywords

estrogen metabolites, 2-methoxyestradiol, uterine artery endothelial cells, pregnancy, angiogenesis, apoptosis

Introduction

Angiogenesis, process of growth of new capillaries from preexisting vessels, is essential for the development and growth of the embryo, the uterus, and the placenta.¹ Angiogenesis is vital for the development of an intricate placental villous vascular tree and the remodeling of maternal uterine spiral arteries leading to the development of a low-resistance and a low-pressure system in human pregnancy.^{2,3} Factors that affect angiogenesis are very likely to alter the gestational rise in uteroplacental blood flow needed for proper maintenance and development of the fetal well-being. The role of 17 β -estradiol (17 β E2) and its metabolites such as 2-methoxyestradiol (2-ME2) in controlling angiogenesis and uterine blood flow in pregnancy has recently been challenged.⁴⁻⁷ We have previously shown that estradiol metabolizing enzymes are expressed in primary ovine uterine artery endothelial cells (UAECs) and that 2-ME2 did not stimulate proliferation of UAECs whereas by contrast 2-hydroxyestradiol and 4-hydroxyestradiol and 4-methoxyestradiol (4-OHE2) were mitogenic at all doses used.⁸ 17 β E2 is metabolized to 2- and 4-OHE2 by cytochrome P450-1A1 and 1A2, respectively. 2-OHE2 is further

metabolized to 2-ME2 by catechol-*O*-methyltransferase (COMT). Therefore, 2-ME2 is a natural metabolite of 17 β E2, albeit with unique properties that are distinctive from its parent compound 2-OHE2. 2-ME2 inhibits angiogenesis, reduces endothelial cell proliferation, differentiation and migration, and promotes apoptosis presumably by causing failure of microtubular polymerization and inhibition of ERK mitogen-activated protein kinase.⁹⁻¹³ 2-ME2 has little estrogen receptor affinity (<1%) and its effects are executed independent of estrogen receptors.¹⁴⁻¹⁶ Recently, Basu and his colleagues using large-scale proteomic analysis have identified many of the novel

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targets for 2-ME2 in pancreatic cancer cells.¹⁷ These signaling pathways include genes associated with apoptosis, cell cycle, DNA repair, and hypoxia.

The unique functions of 2-ME2 in pregnancy have been the focus of recent research. 2-ME2 is essential for normal pregnancy and its deficiency leads to the development of preeclampsia-like symptoms with hypertension and proteinuria in pregnant mice.¹⁸ With the increasing knowledge of the diverse actions of 2-ME2, in particular the recent discovery of its role in normal pregnancy, there remains a gap of knowledge as to how 2-ME2 would induce cell death and angiogenesis on one end and yet be essential for normal placentation and pregnancy on the other end.^{18,19} Herein, we hypothesize that 2-ME2 differentially affect angiogenesis in uterine endothelial cells in the pregnant and nonpregnant state. We investigate the effect of 17 β E2 and 2-ME2 on capillary tube formation and cell proliferation in UAECs from nonpregnant ewes (NP-UAECs) either in the follicular phase (F-UAECs) or in the luteal phase (L-UAECs) or from pregnant ewes (P-UAECs). We showed that pregnancy protected UAECs from the antiangiogenic properties of 2-ME2 as evident by the preservation of capillary tube formation in 2-ME2-treated P-UAEC. This protection was independent of cell apoptosis or microtubule polymerization as there was no difference in either of these 2 parameters between 2-ME2-treated UAECs from pregnant or nonpregnant ewes.

Methods

17 β E2 and 2-ME2 were purchased from Sigma Chemical Co. (St. Louis, Missouri). Rabbit anti-Akt polyclonal antibody (pAb), phospho-specific anti-Akt pAb, phospho-specific anti MAPK polyclonal antibody (pAb), and phospho-specific anti-eNOS pAb were obtained from Cell Signaling (Beverly, Massachusetts). Mouse-specific anti-eNOS monoclonal Ab (N30020) was obtained from BD Transduction Laboratories (Lexington, Kentucky) and Rabbit phospho-specific anti-MAPK pAb from Promega Corp. (Madison, Wisconsin). Secondary antibodies used were donkey antirabbit horseradish peroxidase (HRP)-linked Fab2 from Cell Signaling (Beverly) and sheep antimouse Ig HRP-linked Fab2 (Amersham Pharmacia Biotech). Mouse-specific monoclonal anti- α tubulin antibody was obtained from Sigma, fluorescein isothiocyanate (FITC)-conjugated goat antimouse antibodies from Jackson Immuno Research, ProLong gold antifade reagent from Invitrogen (Grand Island, New York). Tissue culture plastic ware obtained from Corning (Corning, New York). Fetal calf serum (FCS), medium-199 (M-199), phosphate-free DMEM were from Life Technologies Inc. (Grand Island) and Collagenase B from Roche Molecular Biochemicals (Indianapolis, Indiana). Electrophoresis reagents, precasted SDS-PAGE gels were from Bio-Rad Laboratories (Hercules, California). Immobilon-polyvinyl difluoride (PVDF) membrane was from Millipore (Bedford, Massachusetts). Protein concentration of supernatant was determined using bicinchoninic acid assay from Sigma. Enhanced chemiluminescence reagent detection system kits

were from Amersham Pharmacia Biotech (Arlington Heights, Illinois) and super signal west pico chemiluminescence detection system from ThermoScientific (Rockford, Illinois). Signals were analyzed by densitometry using the Imaging Fluorchemtm 8900 from Alpha Innoteck (San Leandro, California). The growth factor reduced Matrigel and Annexin V-FITC Apoptosis detection kits were from BD Biosciences (San Jose, California).

Preparation of Primary Sheep Uterine Artery Endothelial Cells

In vitro primary sheep UAECs have been developed, validated, characterized, and utilized in our laboratory as a feasible model to study uterine vascular endothelial function and angiogenesis. Primary sheep UAECs were established from fresh uterine artery tissues collected from follicular phase ($n = 3$), luteal phase ($n = 3$), and pregnant sheep (days 120-130, $n = 3$) as have been described previously.²⁰ UAECs were studied at passage 4 to 5. Briefly, following nonsurvival surgery, uterine arteries were identified and dissected free of surrounding structures. Uterine arteries were rinsed of blood then the tunica intima was digested using medium 199 containing 5 mg/mL collagenase B (Roche Molecular Biochemicals) and 0.5% BSA at 37°C for 55 minutes. Endothelial cell sheets were flushing from the inner surface of the vessel. This protocol was approved by the University of Wisconsin-Madison research animal care committees of both the School of Medicine/Public Health and the College of Agriculture and Life Sciences and follow the recommended American Veterinary Medicine Association guidelines for euthanasia of laboratory farm animals.

Cell Culture

Freshly obtained UAECs (passage 0) were cultured in T75 flasks in growth media (D-EBM without Phenol Red with 20% FCS, 100 U/mL penicillin, and 100 mg/mL streptomycin), propagated, and frozen at passage 3 in liquid nitrogen for long-term storage. At the time of the experiment, frozen UAECs aliquots were thawed and cultured in 60-mm plates. Approximately 70% confluent monolayers were maintained in media containing 10% charcoal-stripped fetal bovine serum for 24 hours before treating with ethanol (0.001%, vehicle control), 10^{-8} mol/L 17 β E2, or 10^{-9} to 10^{-6} mol/L 2-ME2. Cells were lysed in lysis buffer for protein analysis or were suspended as a single-cell suspension for flowcytometry or fixed on a slide for immunocytochemistry.

Capillary Tube Formation Assay

The growth factor-reduced Matrigel was thawed on ice overnight and diluted with serum-free and Phenol Red-free EBM media. Twelve-well plates were coated with diluted matrigel and incubated at 37°C for 1 hour. A 24-hour serum-starved UAECs monolayer was harvested, cells were counted, and 1×10^5 cells were plated in each well. Based on previous dose-response

studies, UAECs were treated with ethanol (vehicle), 10^{-8} mol/L 17 β E2 or 10^{-6} mol/L 2-ME2. The plates were incubated at 37°C and capillary-like structure formation was evaluated at 6 hours after staining with Calcein-AM or crystal violet (results not shown). Digital images were taken using a Leitz phase-contrast inverted fluorescence microscope at $\times 40$ magnification. The density of the tubes was assessed using Metamorph 6.3 software. Typically, pictures in tif format were opened using metamorph6.3 and the tube formation was quantified for mean tube length, mean tube area, branch points, and connected sets. The branching points were identified as points where the cells join and the tubes branch leading to formation of a node or a clump between the cells. Circular capillary tubes were identified as tubular structures that were surrounded by basement membrane for at least 75% of the diameter. Connected sets indicated capillaries that branch from one node and reconnect to another node.

Protein Quantification and Western Blot

Western blot analyses were performed as described.^{8,20-22} The UAECs were cultured in 6-well plates, serum-starved for 24 hours, and then treated with either vehicle (ethanol), 10^{-8} mol/L 17 β E2 or 10^{-6} mol/L 2-ME2 for 15 minutes for ERK1/2 and 24 hours for Akt and eNOS. The 24-hour time point and duration of treatments were chosen from previous experiments that were done in the lab. These time points were determined to induce maximal activation in previous preliminary time-course studies in our laboratory.²⁰ The cells were washed with ice-cold PBS and lysed in Riba Lysis buffer lysis buffer (Sigma).

The cell lysate was briefly sonicated and centrifuged, and protein concentration of supernatant was determined using the BCA protein assay kit (Thermo Scientific, Rockford, Illinois). The protein samples were heat denatured (95°C, 5 minutes) in Laemmli buffer. Proteins (15-25 μ g/lane) were separated on 10% SDS-PAGE gels and electroblotted on PVDF membrane. The membrane was probed with rabbit phospho-specific (1:3000) or total ERK (1:5000), rabbit phospho-specific (1:1000) or total Akt (1:3000), rabbit phospho-specific (1:4000) eNOS, and mouse-specific (1:750) total eNOS. Secondary antibodies dilutions were 1:2000 to 1:3000 for antirabbit and 1:3000 for antimouse peroxidase conjugated immunoglobulins G (IgGs), respectively. All serum incubations were performed at 4°C overnight for phospho-specific primary antibodies, at room temperature for 2 hours for total primary antibodies, and at 1 hour for the secondary antibodies. The phosphoproteins were visualized by ECL plus detection system (Amersham, Pittsburgh, Pennsylvania) and total proteins were visualized by West Pico detection system (Thermo Scientific, Rockford, Illinois). The immunoreactive signals were quantified by scanning and expressed as phosphoproteins relative to total proteins on the same blot.

Immunocytochemistry and Immunofluorescence Confocal Microscopy

Immunocytochemistry of UAECs was used to assess the effect of 2-ME2 on microtubular polymerization. UAECs were grown

on 4-chambered slides, serum-starved for 24 hours, and treated with either ethanol (vehicle control), 10^{-8} mol/L 17 β E2 or 10^{-6} mol/L 2-ME2 for 24 hours. The cells were then washed with PBS, fixed with 3% paraformaldehyde. The cells were washed with 0.15 mol/l glycine twice to remove excess paraformaldehyde and were then permeabilized with 0.03% Triton X, washed with PBS, and blocked overnight with 5% goat serum. The cells were washed with PBS and incubated for 1 hour with primary mouse monoclonal anti- α tubulin antibody. After washing, FITC-conjugated goat antimouse antibodies was used as a secondary label. The stained slides were mounted using ProLong gold antifade reagent with DAPI and images were taken using confocal microscope at $\times 40$ magnification.

Flowcytometry

Uterine artery endothelial cells were grown in 6-well plates until 80% confluent, serum-starved for 24 hours and treated with ethanol (vehicle) control, 10^{-8} mol/L 17 β E2 or 10^{-9} to 10^{-6} mol/L 2-ME2 for 24 or 72 hours. The cells were harvested, counted, and 1×10^5 cells in 100 μ L volume from each condition in triplicate were stained for Annexin-V using Annexin V-FITC Apoptosis detection kit from BD (BD Pharmingen, cat no 556547). Briefly 5 μ L of FITC-conjugated annexin-V (annexin-V FITC) and 5 μ L of propidium iodide (PI) were added to each tube, vortexed gently, and incubated in the dark at room temperature for 15 minutes. A total of 400 μ L of binding buffer was then added to each tube.²³ Flow cytometric measurements were performed on a FACScan flow cytometer (Becton Dickinson). Emissions were collected in a separate fluorescent channel with doublet-discriminator module turned on. A laser excitation wavelength of 488 nm, using 530/30-nm band pass filter on FL1 was used for FITC and a 585/42 nm band pass filter in FL2 for PI detection. Raw data from were acquired and analyzed with FlowJo software (V. 8.4, TreeStar).

Statistical Analysis

Experiments were typically performed in a minimum of 3 independent triplicates, and when appropriate, the data was analyzed using the 2-tailed Student unpaired *t*-test or 1-way analysis of variance (ANOVA). *P* < .05 was considered statistically significant.

Results

Assessment of Capillary Tube Formation in 2-ME2-Treated Pregnant and Nonpregnant Sheep UAECs

Capillary tube formation assay revealed contrasting effects of 17 β E2 and 2-ME2 on F-UAECs when compared to both L-UAECs and P-UAECs. These data suggest that F-UAECs showed maximum increase of capillary tube formation in response to treatment with 17 β E2 and inhibition with 2-ME2; This was in contrast to L-UAECs which showed minimum

Table 1. Effects of 2-ME2 and 17 β E2 on Capillary Tubes Formation in Uterine Artery Endothelial Cells (UAEC) In Vitro^a

Cell Type	Mean Tube Length μm	Mean Tube Area μm^2	Branch Points	Connected Sets
F-UAECs–Control	3.33 $\pm$ 0.11	13.66 $\pm$ 2.48	13.33 $\pm$ 2.48	50 $\pm$ 3.78
F-UAECs-17 β E2	4.25 $\pm$ 0.33 ^b	23.6 $\pm$ 3.99 ^b	24.7 $\pm$ 10.96 ^b	130 $\pm$ 24.78 ^b
F-UAECs-2ME2	2.76 $\pm$ 0.04 ^b	10.42 $\pm$ 0.98	17.66 $\pm$ 4.04	53.33 $\pm$ 16.01
L-UAECs–Control	3.67 $\pm$ 0.28	23.23 $\pm$ 0.81	30.67 $\pm$ 18.50	159.67 $\pm$ 37.61
L-UAECs-17 β E2	4.12 $\pm$ 0.09 ^b	25.60 $\pm$ 5.97	33.33 $\pm$ 9.45	110 $\pm$ 32.69
L-UAECs-2ME2	3.33 $\pm$ 0.24	22.47 $\pm$ 3.52	23 $\pm$ 13.74	123.67 $\pm$ 27.30
P-UAECs–Control	3.49 $\pm$ 0.22	21.05 $\pm$ 3.31	34.33 $\pm$ 10.69	116.67 $\pm$ 9.29
P-UAECs-17 β E2	3.78 $\pm$ 0.25	23.61 $\pm$ 3.15	20.33 $\pm$ 7.23	92.33 $\pm$ 20.55
P-UAECs-2ME2	3.61 $\pm$ 0.07	18.99 $\pm$ 2.81	40.66 $\pm$ 18.45	109.67 $\pm$ 10.67

^a Capillary tube formation on matrigel was assessed by measurements of mean tube length, mean tube area, branch points, and connected tube sets using Metamorph 6.3 software. F-UAECs, L-UAECs, and P-UAECs were treated for 6 hours with ethanol (0.001%, vehicle control), 10^{-8} mol/L 17 β E2, or 10^{-6} mol/L 2-ME2, stained with Calcein-AM Green Cell Tracker, and imaged by fluorescence microscopy. Data represent the average of 3 experiments.

^b $P < .05$.

Figure 1. In vitro capillary tube formation following treatments of uterine artery endothelial cells (UAEC) with 2-ME2 and 17 β E2. Shown are representative micrographs of capillary tube formation on matrigel from F-UAECs (1a-c), L-UAECs (1d-f), and P-UAECs (g-i) following treatment with ethanol (0.001%, vehicle control), 10^{-8} mol/L 17 β E2, or 10^{-6} M 2-ME2 after 6 hours of incubation. Primary endothelial cells cultures were stained with Calcein-AM Green Cell Tracker and imaged by fluorescence. A network of tubular structure is observed in the control. Addition of 17 β E2 increased capillary tube formation while treatment with 2-ME2 disrupts capillary tube formation by endothelial cells in F-UAEC. The density of the tubular structure and the branching points were more preserved in the presence of 2-ME2 in P-UAECs and L-UAECs when compared to F-UAECs.

response to 17 β E2 and no response to 2-ME2 treatment, and P-UAECs which showed no response to either 17 β E2 or 2-ME2 treatments (Table 1). When compared to controls, 17 β E2 enhanced capillary tube formation in F-UAECs as indicated by increase in mean tube length from 3.33 ± 0.11 to $4.25 \pm 0.33 \mu\text{m}$ after treatment with 17 β E2. 2-ME2 inhibited the tube length in F-UAECs to $2.76 \pm 0.04 \mu\text{m}$ (Table 1, Figure 1a-c); this was significantly lower than control and 17 β E2 treatments, respectively ($P < .05$). There was similar increase with 17 β E2 and decrease with 2-ME2 treatments in mean tube

area (Control 13.66 ± 2.48 , 17 β E2 23.6 ± 3.99 , and 2-ME2 10.42 ± 0.98) in F-UAECs (Table 1).

The branch points and connected sets were also increased after treatment of F-UAECs with 17 β E2, whereas the subtle increase in these 2 parameters with 2-ME2 treatment did not reach statistical significance ($P > .05$). The pattern of response of F-UAECs was different from that noted for other cell types. L-UAECs showed an increase in mean tube length with 17 β E2 treatment and no significant decrease with 2-ME2 treatment (Table 1). Similarly, the mean tube area, branch point, and

Figure 2. Effects of 2-ME2 treatment on microtubule polymerization of uterine artery endothelial cells (UAEC). Shown are representative micrographs of immunocytochemical staining of α -tubulin in UAECs and Ishikawa cells, respectively (also supplemental Figure 1). Cells were treated with ethanol (0.001%, vehicle control), 10^{-8} 17 β E2 or 10^{-6} 2-ME2 for 24 hours. Microtubular assembly and polymerization were assessed by staining for α -tubulin while DAPI and propidium iodide were used to counterstaining of the nuclei. 2-ME2 treatment lead to condensation of tubules around the nucleus and disruption of normal cytoskeletal structure in UAECs (Figure 2. c, f, and i) when compared to controls (Figure 2.a, d, and g), while 17 β E2 had no effect on α -tubulin formation in follicular, luteal or pregnant UAECs (Figure 2.b, e, & h). Tubule condensation was similar to that seen in Ishikawa cells when compared to control (Supplemental Figure 1f and c).

connected sets in L-UAECs did not differ significantly with either 17 β E2 or 2-ME2 treatments when compared to control (Table 1, Figure 1d-f). The pattern of response of the L-UAECs was very similar to P-UAECs. P-UAECs showed no effect to either 17 β E2 or 2-ME2 on any of the capillary tube parameters that were studied (Table 1, Figure 1-i).

Assessment of Microtubule Structure in 2-ME2-Treated Nonpregnant and Pregnant Sheep UAECs

Immunocytochemistry of UAECs cells was used to assess the effect of 2-ME2 on microtubule structure and polymerization. Treatment with 10^{-8} mol/L 17 β E2 (Figure 2A. b, e, and h) had no deleterious effects on microtubule structure and polymerization when compared to control. On the other hand, treatment of P-UAECs with 10^{-6} mol/L 2-ME2 resulted in shrinking of the cells and clustering of the bundles of microtubules around the cell nucleus indicating impaired tubular structure and polymerization when compared to control (Figure 2A). There was no difference in the extent of disruption of microtubule structure and polymerization between follicular, luteal, and pregnant UAECs when cells were treated with 10^{-6} mol/L 2-ME2 (Figure 2A. c, f, and i) as compared to control (Figure 2A. a, d, and g). This is comparable to Ishikawa cells, a glandular

endometrial cell line that is known to undergo apoptosis in response to 2-ME2 (Supplemental Figure 1).

Assessment of Apoptosis in 2-ME2-Treated Nonpregnant and Pregnant Sheep UAECs

Flowcytometry was used to assess apoptosis following 10^{-8} mol/L 17 β E2 and 10^{-9} to 10^{-6} mol/L 2-ME2 treatment. This encompasses the physiologic concentrations for 17 β E2 and 2ME2 (low nanomolar range) as well as that are 10- to 100-fold higher for 2ME2. Treatment of UAECs with 2-ME2-induced endothelial cell apoptosis in a time- and a concentration-dependent fashion. Treatment of UAEC with lower concentration of 2-ME2 (10^{-9} and 10^{-8} mol/L) for 24 hours did not induce cell apoptosis (Figure 3A). When UAECs were treated with higher concentrations of 2-ME2 at 10^{-6} mol/L for 72 hours, a significant number of the cells underwent apoptosis (Figure 3B). Apoptosis was noted to increase equally in both follicular and pregnant UAECs with 2-ME2 treatment while it was not affected by 17 β E2 treatment when compared to control. The percentages of cells undergoing apoptosis among the follicular and pregnant UAECs treatment groups were (0.59 ± 4 and 1.66 ± 2.1) for vehicle control; (4.71 ± 5.8 and 12.14 ± 3.6) for 10^{-8} 17 β E2 treatment; and (62.72 ± 8.5 and 44.5 ± 3.9 , $P \leq .05$ when compared to control and 17 β E2 treatments) for 10^{-6} 2-ME2

Figure 3. Induction of apoptosis following 2-ME2 treatment. Shown are representative graphs of the percentages of cells that are alive or that are undergoing early apoptosis, late apoptosis, or necrosis in response to 17βE2 and either low dose 2-ME2 (10⁻⁸M, Figure 3A) or high dose 2-ME2 (10⁻⁶M, Figure 3B) in uterine artery endothelial cells (UAECs). Uterine artery endothelial cells were cultured, serum-starved for 24 hours, and treated with ethanol (0.001%, vehicle control), 10⁻⁸ mol/L 17βE2, or 10⁻⁶ to 10⁻⁹ mol/L 2-ME2 for 24 to 72 hours. These cells were harvested, stained with propidium iodide and Annexin V for flowcytometry, and analyzed for apoptosis using Flowjo software. 2-ME2 did not induce apoptosis of UAECs from follicular or pregnant sheep following low-dose treatment with 2-ME2 while apoptosis was induced with higher dose of 2-ME2. There was no significant difference in the number of apoptotic cells between control and 17βE2-treated UAECs; however, there was a significant increase in the number of apoptotic cells and decrease in live cells with 10⁻⁶ mol/L 2-ME2 treatment in both follicular and pregnant UAECs. N = 3, * P < .05.

treatment, respectively (Figure 3A and B). There was no difference in the number of cells undergoing necrosis in response to different treatments between the 2 groups.

Effects of 2-ME2 on ERK1/2, Akt, and eNOS Phosphorylation in UAECs From Pregnant and Nonpregnant Sheep UAECs

The effect of 2-ME2 on ERK1/2, AKT, and eNOS phosphorylation was examined in F-UAECs and P-UAECs by Western Blot (Figure 4). There was no significant change in phosphorylation of ERK1/2 following 2-ME2 treatment of F-UAECs or P-UAECs (Figure 4A). Similarly, there was no change in AKT or eNOS phosphorylation following 2-ME2 treatment of F-UAECs or P-UAECs as shown in Figure 4B and C, respectively. There was a trend toward increase in phosphorylation of ERK, AKT, and eNOS with 17 β E2 treatment of F-UAECs when compared to control (Figure 4).

Discussion

Pregnancy is associated with unique hormonal milieu that facilitates the accommodation of the intricate needs of the embryo and the placenta. Successful angiogenesis and the creation of a low-resistance uteroplacental vascular system is a critical feature for normal pregnancy. Factors that affect angiogenesis are likely to affect pregnancy-specific vascular adaptations and maintenance. 2-ME2, an endogenous estrogen metabolite, is of a particular interest in this respect. 2-ME2 is one of the most potent endogenous antiangiogenic and proapoptotic steroids. 2-ME2 is a known inhibitor of endothelial cell proliferation, differentiation, and migration.^{10,11,19} Recently, it was shown that 2-ME2 is essential for regulation of uteroplacental vascular homeostasis in normal pregnancy and that its deficiency in a mouse model was associated with preeclampsia-like symptoms and fetal wastage.¹⁸ This raises the question as to how 2-ME2 would promote apoptosis and inhibit angiogenesis in some tissues and yet be essential for normal placentation and pregnancy on the other hand. In particular, we were interested in investigating whether the unique environment of pregnancy modulates the effects the 2-ME2 on uterine vascular endothelial cell proliferation and differentiation. Therefore, in the current study, we have investigated the effects of 2-ME2 on angiogenesis and proliferation of primary UAECs lines from nonpregnant and pregnant ewes.

We assessed angiogenesis by utilizing 3-dimensional basement membrane assays (capillary tube formation assay on Matrigel) that examines both vascular endothelial cell proliferation and differentiation. We showed that 2-ME2 inhibited capillary tube formation in F-UAECs only and not in L-UAECs or P-UAECs as evident by the preserved density capillary tubular structures and the branching points in L- and P-UAECs in the presence of 2-ME2. Furthermore, we showed that 2-ME2 equally induced apoptosis and microtubular architecture disruption in pregnant and nonpregnant UAECs. Treatment of UAECs with shorter durations of lower doses of 2-ME2 did not

cause cell apoptosis while higher concentrations of 2-ME2 increased apoptosis equally in P-UAECs and NP-UAECs. In addition, higher concentrations of 2-ME2 treatment resulted in microtubule clumping, condensation, and polymerization around the nucleus when compared to 17 β E2 treatment or control. The effect of 2-ME2 on microtubular structure was equally seen in P-UAECs and NP-UAECs. We concluded that the milieu of pregnancy may create a unique pregnancy-specific environment that modulate the action of 2-ME2 on capillary tube formation from UAECs.

We have previously shown that in vitro culture of primary sheep UAECs is a viable model for studying angiogenesis as they closely recapitulates the in vivo signaling events and maintain their memory in regard to their cell of origin.²⁰ We have used this system to study the effects of 17 β E2 on angiogenesis and showed that 17 β E2 plays an important role in endothelial cell proliferation and differentiation. Here, we use this system to investigate the effects of 2-ME2 on UAECs in order to identify possible differential effects of 2-ME2 on capillary tube formation in pregnancy. We show that the inhibitory effect of 2-ME2 on tube formation was less pronounced in UAECs from pregnant and luteal phase sheep when compared to follicular phase.

The precise underlying etiology for the differential action and attenuation of the antiangiogenic effects of 2-ME2 in pregnancy is not apparent. The cellular effects of 2-ME2 are likely dependant on the cell type and the unique cellular milieu in different tissues. Potential factors that may explain the differential action of 2-ME2 in nonpregnant and pregnant endothelial cells include differences in the concentration and metabolism of 2-ME2 in different cell types. Varying concentrations of 2-ME2 were shown to have different effects on cell proliferation and the process of angiogenesis overall. Low doses of 2-ME2 were stimulatory to the proliferation of primary endometrial stroma and Ishikawa cells while higher doses were inhibitory.²⁴ In this study, we show no effects of lower concentrations of 2-ME2 on UAECs, while higher concentrations resulted in cell apoptosis. Another potential factor that could contribute to the differential effects of 2-ME2 on angiogenesis in pregnancy include the distinctive hormonal milieu of pregnancy and the dissimilar expression of hormone receptors, receptor isoforms, and receptors coactivators and corepressors between nonpregnant and pregnant uterine artery endothelium.

Here, we show that the effects of 2ME2 were more pronounced in F-UAECs when compared to L-UAECs and P-UAECs. These effects may be due to progesterone which may play a role in modulating the effects of 2-ME2 on the vascular uterine endothelium in pregnancy. Both pregnant and luteal periods are high progesterone states whereas follicular period is high estrogen state. This difference is further augmented in the sheep model as, unlike the human ovary, the sheep corpus leuteum does not produce any estrogen. The role of progesterone receptors in the regulation of capillary tube formation by 2-ME2 in pregnancy warrants further studies especially in lieu of the current observation as the 2 physiologic states with high progesterone were protected from 2-ME2.

Figure 4. Effect of 2-ME2 on phosphorylation of ERK1/2, AKT, and eNOS in F-uterine artery endothelial cells (UAECs), L-UAECs, and P-UAECs. Cells were serum-starved for 24 hours and were treated with vehicle, 10^{-8} 17βE2, or 10^{-6} 2-ME2 for 10 minutes (ERK1/2) and 24 hours (AKT and eNOS). Proteins were separated on 10% SDS-PAGE and analyzed by immunoblotting with antibodies against phospho- or total proteins. Phosphoprotein bands were determined and were normalized to total protein as described. Values in the histograms are mean $\pm$ SE of 3 separate experiments.

Estrogen receptors (ER) on the other hand are unlikely to play a major role in modulating 2-ME2 action in pregnancy since 2-ME2 has weak affinity for ER. Recently, we have shown that ER- α antagonist has no effects on the proliferation of P-UAECs that were treated with or without 2-ME2 and that neither ER- α or ER- β contributed to the proliferative responses of pregnant UAECs to estrogen metabolites.⁸

The exact signaling cascade for the action of 2-ME2 in uterine artery endothelial cells remains unclear. We have previously shown in our lab that ERK1/2 and AKT phosphorylation signaling pathways are involved in mediating the acute nongenomic action of estradiol in UAEC; so here we wanted to assess if 2-ME2 work through a similar mechanism.²¹ We could not show conclusive evidence of 2-ME2-mediated increase in the acute phosphorylation of ERK1/2, Akt or eNOS in the current model. Our lab is currently reinvestigating the acute nonestrogen-receptor-mediated actions of 2-ME2 via phosphorylation of ERK1/2 and p38 MAPK under extensive time courses (Jobe and Magness, unpublished observation). Many other genes were shown to be regulated by 2-ME2 and could potentially modulate the function of 2-ME2 on UAEC. 2-ME2 inhibited ERK mitogen-activated protein kinase in human adrenal carcinoma cell line.¹² 2-ME2 also inhibited the α -subunit of hypoxia-inducible factor (HIF-1 α) in the murine placenta.^{18,13,25} Other genes that are regulated by 2-ME2 include SMAD that mediates signaling of transforming growth factor β (TGF β), cyclin B1 protein and its associated kinase Cdk1/Cdc2, glucocorticoid receptor, nuclear factor- κ B (NF- κ B), and IRS-1 that promote mitogenesis through activation of the insulin receptor and the insulin-like growth factor I.¹⁷ We have previously shown that higher concentrations of 2-ME2 downregulated Bcl2 and VEGF protein expression in endometrial stroma cells, thus accounting for some of the proapoptotic and antiangiogenic effects of 2-ME2.²⁴ It is possible that the differential regulation of some of these genes by 2-ME2 in uterine vasculature and placental endothelium may alter the effects of 2-ME2 in pregnant and nonpregnant state.

Technical limitations of this study include the fact that 2 different incubation periods were used to assess the effects of 2ME2 on angiogenesis and apoptosis. This was due to the fact that angiogenesis and apoptosis became detected at different time intervals in our cell line. Despite this limitation, our data further reinforce the role of 2ME2 in reproduction. Other data supporting a physiological role of 2-ME2 in reproduction is rather strong. 2-ME2 is totally and exclusively produced by COMT enzyme. COMT^{-/-} mice have shed the light on many of the reproductive roles of 2-ME2.²⁶ Using this model, Zacharia and colleagues have reported that lack of 2-ME2 in COMT^{-/-} mice caused failure to dilate aortic endothelial cells after treatment with 17 β E2 when compared to wild-type littermates indicating that 2-ME2 is the main mediator of the vasodilatory effect of 17 β E2 in aortic endothelial cells.^{6,26,27} Kanasaki and colleagues have shown that 2-ME2 prevented placental clotting, fetal wastage, and preeclampsia in a mice model and that COMT^{-/-} mice that

were deficient in 2-ME2 developed preeclampsia-like symptoms.¹⁸ Furthermore, COMT expression is downregulated in mid-secretory phase that corresponds to the implantation window in human endometrium and is overexpressed in the placenta during the first trimester of pregnancy.^{24,28}

Herein, we show that pregnancy protected P-UAECs from the antiangiogenic effect of 2-ME2, a process that would promote neovascularization and thus elevations in uteroplacental blood flow when the fetal demand for oxygen and nutrients is greatest. Moreover, this has the potential of ensuring that 2-ME2 is not deleterious to angiogenesis of the delicate endothelial cells of the uterine vasculature during pregnancy. Despite the protective effects of pregnancy from the antiangiogenic action of 2-ME2, there were no differences in apoptosis or tubular polymerization between when higher doses of 2-ME2-were used. This may indicate that the processes of angiogenesis and apoptosis in UAEC are independently regulated. If proven, this may further facilitate vascular remodeling process in early pregnancy.

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Declaration of Conflicting Interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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Author Note

Additional efigure can be found at <http://rsx.sagepub.com/content/by/supplemental-data>.

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