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Isolation and Characterization of Ovine Luteal Pericytes and Effects of Nitric Oxide on Pericyte Expression of Angiogenic Factors

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We have demonstrated that vascular endothelial growth factor (VEGF) is expressed in capillary pericytes of the developing corpus luteum (CL) and others have shown that basic fibroblast growth factor (FGF2) and angiopoietins (ANGPT) are present in the CL. VEGF and FGF2 target endothelial cells to initiate angiogenesis and stimulate nitric oxide (NO) production. Conversely, NO may increase VEGF expression by vascular smooth muscle cells and pericytes. To investigate the relationship between these angiogenic factors and NO in the CL, microvascular pericytes and endothelial cells were isolated from CL collected from superovulated ewes ($n = 5$) on d 9 of the estrous cycle. Pericytes were identified by their morphology in culture and by immunofluorescent staining for smooth muscle cell actin. Pericytes were incubated with or without varying doses of the NO-donor DETA-NO for 8 h. Then, total cellular RNA was extracted from the cells and evaluated for expression of mRNA for *VEGF*, *FGF2*, *ANGPT1*, *ANGPT2*, and NO receptor, guanylate cyclase 1, soluble β3 (*GUCY1B3*), using real-time quantitative RT-PCR. NO caused a dose-dependent increase in *VEGF* ($p < 0.001$), *FGF2* ($p < 0.001$), *ANGPT2* ($p < 0.06$), and *GUCY1B3* ($p < 0.03$) mRNA expression. Expression of mRNA for *ANGPT1* in luteal pericytes was not affected by the NO treatment. These data provide further evidence of the role of the luteal pericyte and NO in angiogenic factor expression, and of the potential interactions of pericytes with endothelial cells via NO production.

Key Words: Nitric oxide; angiogenesis; pericytes; corpus luteum.

Introduction

The corpus luteum (CL) is an ovarian structure that grows and regresses during each estrous cycle or pregnancy. Such rapid tissue growth depends on vascular development in order to meet the metabolic demands of the proliferating cells *(1–4)*. Therefore, growth of pericytes and/or endothelial cells in microvascular capillaries during luteal development must be vital to the proper growth and function of the CL *(1,2,5,6)*. In fact, it has been demonstrated that inhibition of vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, results in the suppression of endothelial cell proliferation, restriction of microvascular development, and decreased progesterone production, indicating that this angiogenic factor is capable of influencing vascular structure and the overall function of the developing CL *(7–9)*.

The mature CL is highly vascularized, with 50–70% of the tissue comprised of microvascular pericytes and endothelial cells *(1,2,10)*. Pericytes are mesodermally derived cells that wrap around the outside of capillaries and are separated from endothelial cells by a basement membrane. Pericytes are of vascular smooth muscle cell (SMC) lineage and are therefore similar to vascular smooth muscle cells *(11)*. Cytoplasmic projections of the pericyte may protrude through the basement membrane to allow for direct contact between pericytes and endothelial cells *(12,13)*. It has been suggested that interactions between pericytes and endothelial cells are important for maintaining functional neovascularization in numerous organs and tissues *(2,14,15)*. Numerous regulatory factors, produced by pericytes and endothelial cells within the microvasculature such as nitric oxide (NO), VEGF, basic fibroblast growth factor (FGF2) or angiopoietins (ANGPT), can stimulate the process of angiogenesis *(16,17)*. Nitric oxide is known to mediate physiological functions, such as vasodilation, angiogenesis, and blood flow in many tissues including the ovary *(18,19)*. Two major angiogenic factors produced by the CL are VEGF and FGF2 *(2,9,10,16)*. We have previously demonstrated that VEGF is expressed in pericytes of the ovine CL *(10)*. Furthermore, we and others have demonstrated the presence of FGF2,

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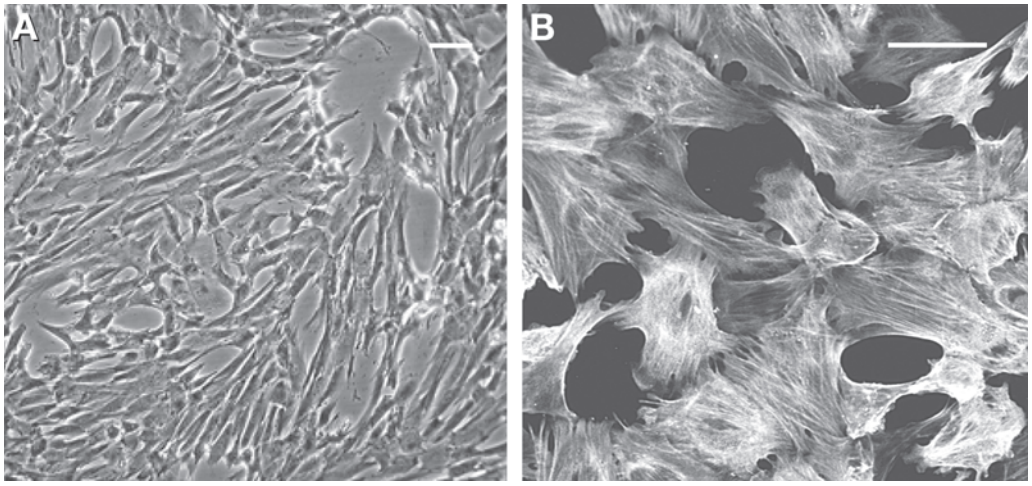

Fig. 1. Representative micrographs of ovine luteal pericytes ($n = 5$ ewes; $n = 1$ culture/ewe) in culture (**A**, bright-field, bar = 38 μm) and immunofluorescent staining of pericytes for smooth muscle cell actin (**B**, bar = 38 μm). Note: bright white immunofluorescent staining positively identifies pericytes.

ANGPT1, and ANGPT2 during CL growth, differentiation, and regression *(9,20–22)*.

We hypothesized that NO regulates the expression of several major angiogenic factors and the receptor for NO, guanylate cyclase 1, soluble, β3 (*GUCY1B3*) in luteal pericytes. Therefore, the aims of the present study were two-fold: (1) to isolate, characterize, and maintain pure cultures of pericytes from the CL and (2) to investigate NO-induced effects on expression of mRNA for *VEGF*, *FGF2*, *ANGPT1, ANGPT2,* and the NO receptor, *GUCY1B3* in luteal pericytes in vitro.

Results

Isolation of Pure Cultures of Luteal Pericytes

Pericytes ($n = 5$ cultures from five ewes) were successfully isolated from mature CL by a multistep procedure, which included (1) enzymatic digestion of luteal tissues, (2) separation of endothelial cells from a mixed luteal cell population using magnetic beads coated with lectin from *Bandeiraea simplicifolia (Griffonia simplicifolia*; BS-1), and (3) separation of the remaining cells using a Percoll gradient to obtain fractions enriched with non-steroidogenic cells, small luteal cells, or large luteal cells. Then, fractions containing non-steroidogenic cells and/or small luteal cells were cultured separately. Cells were grown to near confluence, and then passaged four times. At each passage, cells were stained for the presence of specific markers of pericytes/smooth muscle cells, endothelial cells, and steroidogenic cells.

Immunocytochemistry and Acetylated Low-Density-Lipoprotein (LDL) Uptake

Isolated luteal pericyte cultures were successfully established and each passage was substantially identified as pericytes with a specific marker (described below), and by their morphology (Fig. 1A), which was similar to the in vitro pericyte morphology described by others *(11,12,23)*. Smooth muscle cell actin (SMCA), a pericyte/SMC marker (*10*), was detected in the cytoplasm of luteal pericytes (Fig. 1B). Pericyte cultures exhibited no positive staining for markers of other cells, including: (1) fibroblasts (collagen type 1; *(24,25)*, (2) steroidogenic cells (3β-hydroxysteroid dehydrogenase [3β-HSD]) *(26)*, and (3) endothelial cells (BS-1 lectin, factor VIII, angiotensin converting enzyme and rapid uptake of LDL) *(10,27,28*). Examples of positive staining of ovine luteal tissues or cells for SMCA, 3β-HSD, and collagen type 1 have previously been published by our laboratory *(10,25,26)*. The absence of positive staining for the markers of other cell types (fibroblasts, endothelial, and steroidogenic cells), along with distinct positive staining for the pericyte marker SMCA, demonstrated that these pericyte cultures were not contaminated by other cells. Aortic smooth muscle cell cultures, which abundantly express SMCA, were used as positive controls for SMCA (Fig. 2A), whereas ovine luteal and bovine aortic endothelial cell were used as positive controls for factor VIII, BS-1 lectin, angiotensin-converting enzyme, and rapid uptake of LDL (Figs. 2B–E, respectively). Because the pattern of staining for factor VIII, BS-1 lectin, angiotensin-converting enzyme, and rapid uptake of LDL was similar for ovine luteal and bovine aortic endothelial cells, only data for luteal endothelial cells are shown (Figs. 2B–E). Moreover, morphology of pericytes cultures was stable across passages. Greater than 95% of the cells were SMCA positive, and negative for markers of fibroblasts, steroidogenic, and endothelial cells at every passage and level of confluency, thus further confirming the purity of the pericyte cultures.

NO Levels and NO Donor (DETA-NO) Treatment

This preliminary experiment was conducted to determine the best procedure for generating NO in cell culture. Ovine

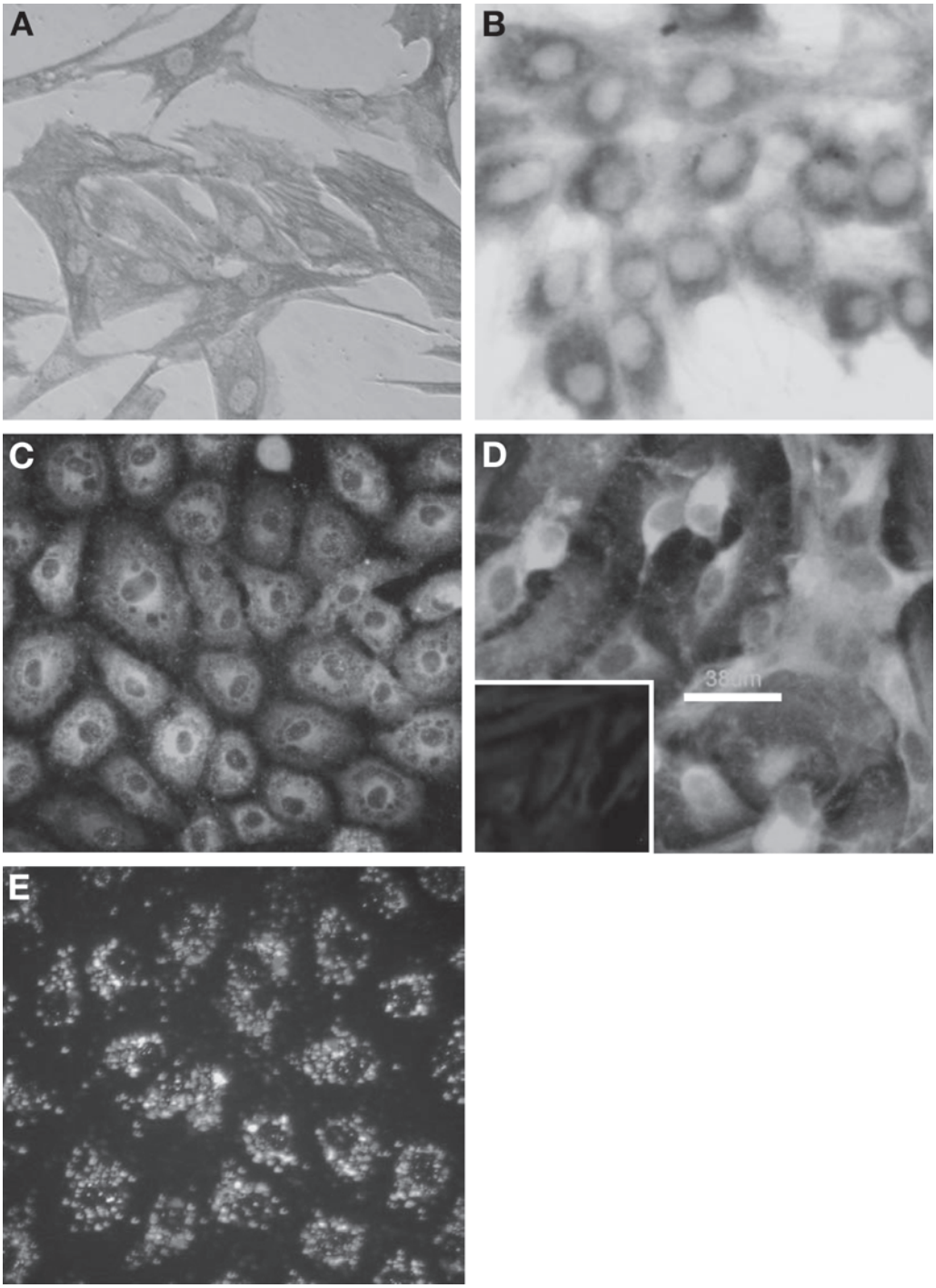

Fig. 2. Representative micrographs of (**A**) SMCA in ovine aortic smooth muscle cells, (**B**) lectin BS-1, (**C**) factor VIII, and (**D**) ACE positive staining (black color in **A** and **B**, respectively; and bright white fluorescence in **C** and **D**) in ovine luteal endothelial cells isolated with BS-1 coated magnetic beads. (**E**) BS-1 positive luteal endothelial cells demonstrating LDL uptake (bright white fluorescence indicates LDL uptake). Inset in **D** shows lack of staining of pericytes for the presence of factor VIII; a similar lack of staining was observed for BS-1 lectin and ACE (data not shown). Pericyte cultures ($n = 5$) were consistently negative for these markers at each passage. For all micrographs, representative bar shown in **D** = 38 μm.

aortic SMC were selected because this cell type is known to be responsive to NO *(29,30)* and these cells were obtained from sheep *(31)*. When aortic SMC were cultured for 8 h in the presence of NO donor, DETA-NO, the NO concentrations in the culture media at the end of culture (Fig. 3, top panel) were similar to the predicted NO concentrations (based on predicted generation of 2 moles of NO for every mole of DETA-NO) for the first three doses of DETA-NO, but were slightly less than predicted for the final 10.0 μM dose of DETA-NO. In addition, when aortic smooth muscle cells

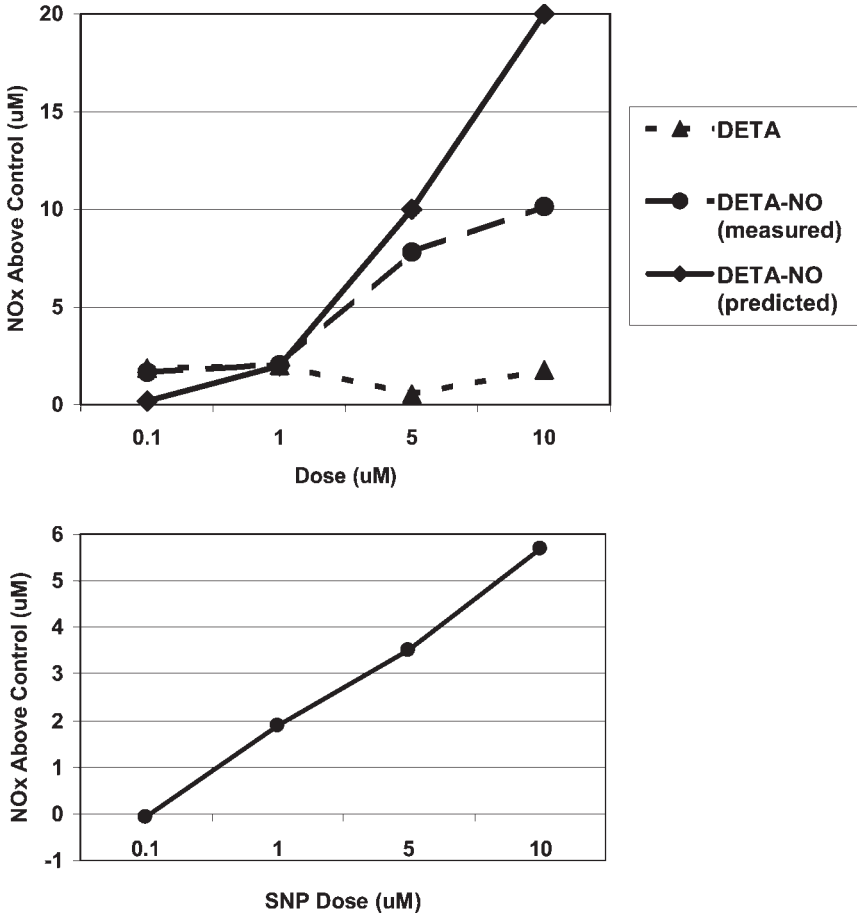

Fig. 3. NO concentrations in culture media from (Top panel) aortic vascular smooth muscle cells cultured (n = 3/treatment) for 8 h in the presence of DETA or DETA-NO along with the NO predicted concentration. (Bottom panel) Aortic smooth muscle cells cultured (n = 3/treatment) for 8 h in the presence of sodium nitroprusside (SNP). Concentrations of NO in the absence of either DETA, DETA-NO or SNP were undetectable.

were cultured for 8 h in the presence of another NO donor, sodium nitroprusside, NO concentrations in the media at the end of culture were about half those seen for DETA-NO (Fig. 3, bottom panel). Thus, although the actual NO concentrations in media from cells cultured in the presence of DETA-NO for 8 h were slightly less than predicted, they were more consistent with the predicted NO concentrations than the cells cultured in the presence of sodium nitroprusside, which justified our use of DETA-NO as the NO donor in this experiment. The 8 h time course for the DETA-NO treatment of pericytes was based on a preliminary study demonstrating that DETA-NO treatment of sheep aortic SMC produced a greater increase in VEGF mRNA expression after 8 than after 12 or 24 h *(31)*.

Expression of mRNA for Angiogenic Factors and GUCY1B3 *in Luteal Pericytes Exposed to DETA-NO*

Expression of the mRNA for *VEGF, FGF2, ANGPT1, ANGPT2,* and *GUCY1B3* was detected in luteal pericytes. Expression of the mRNA for *VEGF, FGF2, ANGPT2,* and *GUCY1B3* was enhanced in the presence of higher doses of DETA-NO (Fig. 4). Treatment with DETA-NO caused a 10- to 15-fold increase ($p < 0.001$) in *VEGF* mRNA expression and a two- to eightfold increase ($p < 0.001$) in *FGF2* mRNA expression, 40- to 50-fold ($p < 0.06$) increase in *ANGPT2* mRNA expression and approx 3.5-fold ($p < 0.03$) increase in *GUCY1B3* mRNA expression compared with the DETA control treatment in luteal pericytes. However, DETA-NO did not affect the expression of mRNA for *ANGPT1*. The variation in amount of RNA used in RT-PCR reactions as determined by amount of 18s RNA expression was minimal and did not differ between luteal pericyte cultures or across doses of DETA or DETA-NO ($p > 0.33$ for DETA; $p > 0.53$ for DETA-NO).

Discussion

We have described a new method that allows to establish pure cultures of microvascular pericytes from the CL. To our knowledge, we are the first to isolate pericytes from the CL and successfully maintain them in cell culture. Additionally, we used a representative culture of these pericytes for experiments designed to evaluate the functional roles of

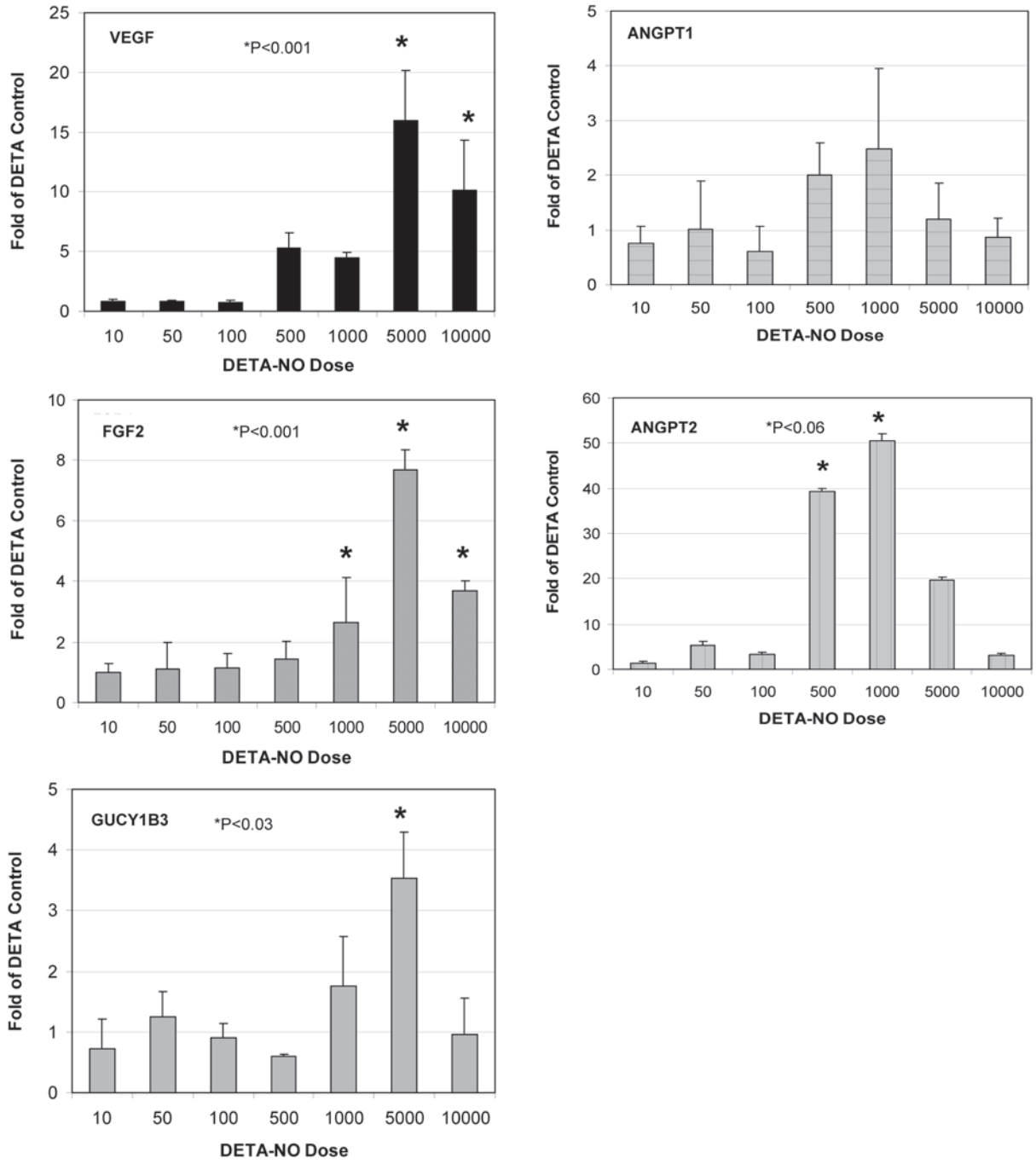

Fig. 4. Dose-response effects of DETA-NO (μM; $n = 3$ treatments/culture) on *VEGF*, *FGF2*, *ANGPT1*, *ANGPT2,* and *GUCY1B3* mRNA expression in ovine luteal pericytes. Data are expressed as a fold increase of DETA control. *Values differ from controls. Error bars = SEM.

pericytes in angiogenesis in the CL. Thus, in this article we have also presented new information on the effect of NO on luteal pericyte expression of mRNA for several major angiogenic factors, namely, *VEGF, FGF2, ANGPT1,* and *ANGPT2,* and for the NO receptor, *GUCY1B3*.

In the CL, pericyte proliferation occurs primarily during the early phase of the estrous cycle, the time when most angiogenesis occurs, and then decreases dramatically during the late luteal phase *(10)*. Thus, when new blood vessels are being formed in the CL, pericytes are being recruited to the outer wall of the vessel to stabilize the new vessels formed *(8)*. These pericytes and vascular smooth muscle cells have been identified as the primary cells that express VEGF and thus stimulate angiogenesis in the CL *(10)*. Numerous other studies also demonstrated that vascular smooth muscle cells produce and/or express VEGF in several species *(32,33)*. However, species-dependent differences in expression of VEGF by specific luteal cell types exist, because, in contrast to ovine CL, VEGF was localized to steroidogenic cells in the CL of other species *(8,9)*.

Angiogenesis is associated with vasodilation, or relaxation of the vascular wall, a process that is mediated by NO *(34,35)*. The present study demonstrates that the NO-donor DETA-NO caused a dramatic dose-dependent increase in *VEGF* mRNA expression in luteal pericytes. This indicates that NO can regulate *VEGF* expression in pericytes and further solidifies a proposed paracrine loop between VEGF expressed by pericytes and NO from endothelial cells and/or other cells in angiogenesis *(2,15,17,36,37)*. Nitric oxide effects on VEGF expression have also been reported for other cell types such as macrophages, keratinocytes, and tumor cells *(33)*. This indicates that interactions between NO and VEGF are not limited to luteal tissues, but are present in other tissues as well.

Our study also demonstrated that NO caused a dose-dependent increase in *FGF2* mRNA expression in luteal pericytes. FGF2 has been shown to protect cells from undergoing apoptosis and has been proposed as a survival factor for preventing luteal regression *(2,38,39)*. Thus, the increase in expression of *FGF2* mRNA by luteal pericytes may be a protective response to the increasing levels of the free-radical NO. Additionally, FGF2 has been shown to stimulate pericyte migration, cell division, and VEGF expression in ovarian and other tissues *(2,40,41)*. The cessation of angiogenesis is coincident with the migration of pericytes to the growing vessel; therefore, this increase in *FGF2* mRNA in luteal pericytes mediated by NO may be associated with the termination of angiogenesis *(14)*. Because FGF2 has pleiotropic effects on a mixture of luteal cells during CL development, we suggest that increased expression of *FGF2* mRNA may also play a role in mediating the interaction between NO and VEGF *(2,9,16,42)*.

In this study, there was no change in *ANGPT1* mRNA expression by luteal pericytes after NO treatments. Because NO treatment did not effect expression of *ANGPT1* mRNA by pericytes, it seems that, in the differentiated ovine CL, pericytes are not stabilized by *ANGPT1* mRNA, or that pericytes must be cultured with endothelial cells for *ANGPT1* mRNA expression to be affected by NO.

ANGPT2 is thought to be an antagonist to ANGPT1 due to its association with vessel destabilization *(9,43)*. In this study, we have demonstrated increase in *ANGPT2* mRNA expression in luteal pericytes exposed to NO donor. The increased *ANGPT2* mRNA expression by pericytes suggests that ANGPT2, rather than ANGPT1 may be involved in the regulation of luteal angiogenesis *(43)*. As the CL mature, VEGF expression decreased but the levels of ANGPT2 remained elevated *(10,21,22)*. When luteal *VEGF* mRNA expression decreases, increased *ANGPT2* mRNA expression by pericytes may allow for vessel destabilization in order to trigger luteal regression near the end of the estrous cycle *(21)*.

The present data demonstrated that NO may increase expression of *GUCY1B3* mRNA by luteal pericytes. Furthermore, others have reported that pericytes respond to NO with increased intracellular levels of cyclic GMP, which is mediated by GUCY1B3 *(23)*. In fact, it has been demonstrated for several tissue and cell types that NO regulates its own receptor by increasing or decreasing GUCY1B3 expression *(44)*. Our data indicate that in luteal pericytes NO can exert its effects by up-regulating GUCY1B3 expression.

In summary, we have successfully isolated and maintained luteal pericytes in culture and we have shown that NO treatment in vitro caused a significant increase in mRNA expression of *VEGF*, *FGF2, ANGPT2,* and the NO receptor, *GUCY1B3*, but not *ANGPT1*. Because NO is primarily produced by luteal endothelial cells *(5)*, we suggest that there is a complex set of paracrine interactions that occurs among pericytes and other cell types (e.g., endothelial and steroidogenic cells) in luteal tissues that is mediated by the NO signaling pathway, and likely by other mechanisms. In future studies, we will use pericytes, endothelial, and steroidogenic cell cultures and co-cultures to evaluate and understand the positive and negative feedback loops involving NO and angiogenic factors that are involved in the regulation of vascular function during growth, differentiation, and regression of the CL.

Materials and Methods

Animals

All animal procedures were approved by the NDSU Institutional Animal Care and Use Committee. Ovaries were obtained from superovulated ewes ($n = 5$) *(26)* on d 9 of the estrous cycle to collect fully differentiated CL, which produces large amounts of progesterone and has a fully developed vascular network *(27)*. Day 9 represents the mid-luteal phase of the estrous cycle; the normal duration of the estrous cycle is 16–17 d for our sheep flock.

Isolation of Pure Cultures of Luteal Pericytes

To prepare for removal of endothelial cells from dispersed luteal cells, magnetic tosylactivated beads (Dynabeads M-450, Dynal Inc., Lake Success, NY) were coated with BS-1 lectin 1 (Vector Laboratories, Burlingame, CA) following the manufacture's directions and stored at 4°C. BS-1 has been used to separate endothelial cells from other cell types in a variety of tissues because it binds specifically to endothelial cells *(10,45,46)*.

Luteal tissues were dispersed using collagenase digestion (Type IV; Worthington, Freehold, NJ) as previously described *(26)*. After completion of digestion, the luteal cells were filtered through a sterile 55-μm nylon mesh and resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). To remove endothelial cells, BS-1–coated beads were added to the monodispersed cells in PBS + 1% BSA at a ratio of 20 beads per cell and mixed for 25 min at 4°C on a rocking platform. The cell/bead solution was placed in front of a magnet and the bead-free supernatant was removed and used for isolation of pericytes

using density gradient centrifugation. Luteal endothelial cells (BS-1 lectin positive) were then cultured in 35 mm Petri dishes coated with fibronectin (10 μg/mL of PBS; Gibco, Grand Island, NY) in a selective medium comprised of Dulbecco's modified Eagle's medium (DMEM), plasma-derived horse serum (20%; Hyclone, Logan, UT), penicillin–streptomycin (1%; Gibco), endothelial cell growth supplement (1 mg/mL; Collaborative Biomedical Products, Bedford, MA), heparin (100 μg/mL; Sigma, St. Louis, MO), and D-valine (73 μg/mL; Sigma) without removing the beads. After reaching confluence, cells were plated in four-chamber slides and used as controls for immunocytochemical staining, as described below.

The endothelial cell–free fraction was layered on top of a Percoll™ (Amersham Biosciences, Piscataway, NJ) gradient (70%, 50%, 40%, and 20%), and centrifuged for 15 min at 500*g* to isolate cell fractions. After centrifugation, each gradient was removed separately from the tube, DMEM added, and centrifuged again at 200*g* for 10 min. Pericytes were isolated from the fraction containing 50% Percoll. The isolated cells were suspended in DMEM supplemented with 10% calf serum (Gibco) and 1% penicillin–streptomycin, counted on a hemocytometer, and then plated into 35 mm dishes. Pericytes were grown to near confluence at 37°C in a humidified incubator containing 5% CO_2 and 95% air and then split into other dishes. Cells were passaged in this manner four times before being frozen (2×10^6 cells/mL/cryovial) in liquid nitrogen as we have previously described *(28)*. Cell passages were necessary to provide the number of cells needed for functional studies and to freeze aliquots for future experiments.

The newly isolated 50% Percoll fraction contained from 100,000 to 500,000 cells per CL; after four passages and before freezing, the total number of pericytes increased to 2 to 5 million per CL. The number of CL per ewe ranged from 6 to 24 CL.

Immunocytochemistry

To confirm that isolated cells from five sheep were pericytes and were free from contamination by other cell types, immunofluorescent staining using markers for specific cell types was conducted as follows: for pericytes, SMCA was used *(10)*; for steroidogenic cells, 3β-HSD was used *(26)* for fibroblasts, collagen type I was used *(24,25)*; and for endothelial cells, factor VIII-related antigen *(27),* BS-1 *(10)*, ACE, and rapid uptake of LDL were used *(28)*. Ovine aortic SMC, luteal endothelial cells, and bovine aortic endothelial cells *(28)* were used as positive or negative controls. Briefly, each cell type was grown in several four-well chamber slides (Lab-Tek® Permanox; Nalge Nunc International Corp., Naperville, IL) for 2–4 d in DMEM containing 20% serum (Gibco). After reaching confluence, the media was discarded and the cells were rinsed three times using PBS to remove traces of serum. The cells were fixed for 15 min using a solution of 85% ethanol and 15% glacial acetic acid. The fixed cells then were washed in 70% ethanol followed by a wash in PBS containing 0.3% (v/v) Triton X-100, a wash in PBS alone, and an incubation in 1% (v/v) normal goat serum (Vector Laboratories). This was followed by overnight incubation at 4°C with a specific primary antibody to factor VIII (Sigma Chemical Co., St. Louis, MO), angiotensin-converting enzyme (Chemicon, Temecula, CA), SMCA (Sigma), and collagen type I (Calbiochem/EMB Biosciences, San Diego, CA). Visualization of the primary antibody was accomplished using a fluorescein isothiocyanate–conjugated secondary antibody (Alexis, San Diego, CA) or biotin-labeled secondary antibody and AEC substrate kit (Vector Laboratories). Control staining consisted of replacing the primary antibody with the same dilution of rabbit serum or mouse serum. To detect BS-1 lectin binding, cells were fixed as described above and then were incubated with BS-1 lectin (Sigma; 12.5 μg/mL) followed by incubation with an avidin and biotinylated horseradish peroxidase macromolecular complex according to the manufacturer's recommendations (Vectastain Elite ABC Reagent, Vector Labs) *(10)*. Colorimetric visualization was completed by using SG substrate (Vector Labs). To rule out contamination of pericyte cultures with steroidogenic cells, cells were fixed in 10% formalin solution for 20 min followed by incubation with 3β-HSD staining solution, as described before *(26)*. Steroidogenic cells stain positive for 3β-HSD, whereas pericytes are negative. Additionally, to rule out contamination of cultures with endothelial cells, assays for uptake of fluorescently labeled 1,1'-dioctadecyl acetylated LDL (DiI-Ac-LDL; Biomedical Technologies, Stoughton, MA) were performed as previously described *(28)*. Endothelial cells demonstrate rapid uptake of LDL, whereas pericytes do not. A Nikon Microphot microscope equipped with a Nikon DXM 1200 digital camera was used to take all photomicrographs (except for those of culture morphology) using bright field, phase-contrast, or fluorescence microscopy. A Nikon Eclipse TE 300 inverted microscope equipped with a Nikon Coolpix 990 digital camera was used to take micrographs of cultured cells.

Treatment with NO Donor

Out of five similar pericyte cell cultures obtained from five different sheep, we randomly chose pericytes from one representative culture to perform this analysis. Pericytes were thawed and grown to confluence for 7 d in DMEM supplemented with 10% CS and 1% penicillin/streptomycin antibiotics in T-75 flasks (Becton Dickinson, Franklin Lakes, NJ). The cells were then plated on 60 mm Petri dishes (5.0×10^5 cells per dish) and cultured for 72 h in the same media. Media were changed to DMEM with antibiotics 12 h before the desired dose of NO donor stock solution (Z)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate (DETA-NO; Cayman Chemical, Ann Arbor, MI) or the control stock solution of diethylenetriamine (DETA; Sigma) were added to the plates ($n = 3$ per

Table 1
Sequence of Sheep-Specific TaqMan Primers and Probes

Oligonucleotide[a]	Nucleotide Sequence	Accession number[b]
VEGF FP	5'-GGA TGT CTA CCA GCG CAG C-3'	X89506
VEGF RP	5'-TCT GGG TAC TCC TGG AAG ATG TC-3'	
VEGF probe	5'(6FAM)-TCT GCC GTC CCA TTG AGA CCC TG-(TAMRA)3'	
FGF2 FP	5'-CGA CGG CCG AGT GGA C-3'	L36136
FGF2 RP	5'-CTC TCT TCT GCT TGA AGT TGT AGT TTG-3'	
FGF2 probe	5'(6FAM)-TCC GCG AGA AGA GCG ACC CTC AC-(TAMRA)3'	
GUCY1B3 FP	5'-CCG AGC CGT GCA TCC A-3'	AF486295
GUCY1B3 RP	5'-ATC TCC ATC ATG TCC AAG GCC-3'	
GUCY1B3 probe	5'(6FAM)-CAT GCA CGG TCC ATC TGC CAC C-(TAMRA)3'	
ANG-1 FP	5'-AAA TGA AAA GCA GAA CTA CAG GTT GTA T-3'	*
ANG-1 RP	5'-GCA AGA TCA GGC TGC TCT GTT-3'	*
ANG-1 probe	5'(6FAM)-TGA AGG GTC ACA CTG GGA CAG CAG G-(TAMRA)3'	*
ANG-2 FP	5'-AAA TAG GGA CCA ACC TGC TCA A-3'	*
ANG-2 RP	5'-TGT TGT CTG ATT TAA TAC TTG GGC TT-3'	*
ANG-2 probe	5'(6FAM)-CTG CAG AGC AGA TCC GGA AGT TAA CAG ATG T-(TAMRA)3'	*

[a]FP, Forward primer and RP, reverse primer.
[b]Nucleotide sequences for ovine-specific genes were obtained from the National Center for Biotechnology Information (NCBI, 2003) database.
*Partial sequences for primer design were graciously provided by Drs. Russell Anthony and Timothy Regnault (Perinatal Research Center, Department of Pediatrics, University of Colorado Health Sciences Center, Denver, CO).

dose). Doses of DETA-NO or DETA were as follows: 0 μM (control), 10 μM, 50 μM, 100 μM, 500 μM, 1 mM, 5 mM, and 10 mM. Following the 8-h incubation with treatments, media were discarded and mRNA from each plate was harvested using Tri-Reagent as recommended by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH). After isolation, RNA from three plates per treatment was pooled. A total of three separate trials were conducted for this study.

We chose to use DETA-NO based on its stability and on previous reports in which it has been shown to stimulate VEGF expression in a number of different cell lines *(47–49)*.

NO Analysis

To ensure that the level of NO to which the pericyte cultures were exposed was similar to the reported NO release rate of 2 moles of NO per mole of DETA-NO, we conducted an experiment using ovine aortic smooth muscle cells exposed to DETA-NO for 8 h. The aortic smooth muscle cells were isolated from ovine aortas and also were identified using the same procedures as described above for ovine luteal pericytes. As a positive control, additional aortic smooth muscle cells cultures were exposed to sodium nitroprusside, a more commonly used NO donor. All cultures and treatments were performed in triplicate. Media was collected at the end of culture and was stored at –80°C until analyzed for NO.

NO was analyzed as described by Magness et al. *(50)* and Rupnow et al. *(51)*. Briefly, a 500 μL media sample was added to 1 mL chilled 100% ethanol, vortexed, and centrifuged at 12,000g for 5 min. The supernatant was analyzed using a Seivers Instruments model 280 NO analyzer (Boulder, CO). Supernatant samples were injected into the purge vessel where nitrates and nitrites in the sample react with V_3Cl_3 to produce NO. The NO gas then flows into the NO analyzer where it reacts with ozone to produce nitrite, which is then quantified by luminescence. The area under the peak was calculated and the amount of NO in the sample was determined by comparing against the standard curve.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Procedures for determining expression of mRNA for angiogenic factors in ovine tissues have been reported previously *(52)*. Briefly, the quality and quantity of total cellular RNA from pericyte cultures were determined via capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). Real-time RT-PCR reagents, probes, and primers were purchased from and used as recommended by Applied Biosystems (Foster City, CA). For each sample, 30 ng total RNA was reverse transcribed in triplicate using random hexamers. Sequence-specific Taqman probes and primers sets (shown in Table 1) were developed using Primer Express *(52)*. Each amplicon, as well as the primers, and probes were shown to be species-specific for the gene of interest by NCBI BLAST. The ABI PRISM 7000 was used for detection of sequences amplified at 60°C for 40 cycles (Applied Biosystems). Quantification was determined from a relative standard curve of varying doses of a

reference standard cDNA generated from tcRNA pooled from placentomes collected at d 130 of pregnancy. To control for variations in the amount of RNA used, individual pericyte cultures were also analyzed for concentrations of 18S RNA using the 18S PDAR kit reagents from Applied Biosystems.

Statistical Analysis

Within each treatment (DETA-NO or DETA), the RT-PCR value from each trial was normalized to its untreated control. Then, these normalized values for the DETA-NO treatments were divided by the normalized DETA control values to calculate the fold response to the DETA-NO treatment. The fold-response values were then analyzed using the general linear model procedure of SAS *(53)*. When the *F*-test was significant, differences between the zero dose and specific doses were compared using least significant differences test *(54)*.

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