Expression of endothelial nitric oxide synthase in the ovine ovary throughout the estrous cycle

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Abstract

This study was conducted to evaluate the expression of endothelial nitric oxide synthase (eNOS) in ovarian follicles and corpora lutea (CL) throughout the estrous cycle in sheep. Three experiments were conducted to (1) immunolocalize eNOS protein, (2) determine expression of mRNA for eNOS and its receptor guanylate cyclase 1 soluble β3 (GUCY1B3), and (3) co-localize eNOS and vascular endothelial growth factor (VEGF) proteins in the follicles and/or CL throughout the estrous cycle. In experiment 1, ovaries were collected from ewes treated with FSH, to induce follicular growth or atresia. In experiment 2, ovaries were collected from ewes treated with FSH and hCG to induce follicular growth and ovulation. In experiment 3, ovaries were collected from superovulated ewes to generate multiple CL on days 2, 4, 10, and 15 of the estrous cycle. In experiments 1 and 2, the expression of eNOS protein was detected in the blood vessels of the theca externa and interna of healthy ovarian follicles. However, in early and advanced atretic follicles, eNOS protein expression was absent or reduced. During the immediate postovulatory period, eNOS protein expression was detected in thecal-derived cells that appeared to be invading the granulosa layer. Expression of eNOS mRNA tended to increase in granulosa cells at 12 and 24 h, and in theca cells 48 h after hCG injection. In experiment 3, eNOS protein was located in the blood vessels of the CL during the estrous cycle. Dual localization of eNOS and VEGF proteins in the CL demonstrated that both were found in the blood vessels.

Introduction

Nitric oxide (NO) is known to mediate physiological functions, such as vasodilation, regulation of angiogenesis, and blood flow in many tissues, including the ovary (Maylan 1999, Fukumura et al. 2001, Cooke 2003, Duda et al. 2004). The major regulator of NO production is the enzyme, NO synthase (NOS), which appears in three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS; Rosselli et al. 1998, Dixit & Parvizi 2001). Both eNOS and iNOS have been detected in ovarian tissues of several species (Olson et al. 1996, Zackrisson et al. 1996, Jablonka-Shariff & Olson 1997, Al-Gubory et al. 2005). One of the major regulators of eNOS expression and NO production is vascular endothelial growth factor (VEGF), a potent angiogenic and vascular permeability factor (Maylan 1999, Reynolds et al. 2000, 2002). In turn, NO regulates the expression of VEGF and angiogenesis (Reynolds et al. 2000, 2002, Beckman et al. 2006).

During each estrous cycle, ovarian follicles or corpora lutea (CL) exhibit periodic and dynamic growth and regression. Follicles and the CL are highly vascular ovarian tissues, relying on angiogenesis and regulation of blood vessel function for their normal function (Redmer & Reynolds 1996, Reynolds et al. 2000, 2002, Augustin 2001). In follicles, the growth of the inner network of capillaries in the theca interna coincides with a period of rapid growth and differentiation (Augustin 2001, Reynolds et al. 2000, 2002). In fact, increases in vascularity may influence the selection of a dominant, ovulatory follicle, whereas reduced vascularity may lead to follicular atresia (Redmer & Reynolds 1996, Augustin 2001, Reynolds et al. 2002). In addition, reduction in the vascularity of follicles may decrease oocyte quality (Borini et al. 2001, Mercé et al. 2006). After ovulation, dramatic growth and vascularization of the ovulated follicle occur, transforming its structure into a developing CL. The primary function of the CL, production of progesterone, is accompanied by the development of an extensive vascular system, which becomes maximally dilated (Wiltbank et al. 1990, Reynolds et al. 2000, Augustin 2001). In fact, the mature CL, one of the most vascularized tissues of the body, receives most of

eNOS was detected in ovarian follicles and the CL during the estrous cycle in several species (Rosselli et al. 1998, Maul et al. 2003, Al-Gubory et al. 2005). It has been demonstrated that NO plays a role in the regulation of angiogenesis, steroidogenesis, apoptosis, and luteolysis (Rosselli et al. 1998, Al-Gubory et al. 2005, Skarzynski et al. 2005, Weens et al. 2005). However, the expression of eNOS across all stages of follicular and luteal development has not been evaluated in detail. Therefore, these experiments were designed to (1) immunolocalize eNOS protein, (2) determine expression of mRNA for eNOS and its receptor guanylate cyclase 1 soluble β3 (GUCY1B3), and (3) co-localize eNOS and VEGF proteins in follicles and/or CL throughout the estrous cycle.

Materials and Methods

The protocols and animal care for this study were approved by the Institutional Animal Care and Use Committee at NDSU. Crossbred ewes that exhibited at least one estrous cycle of normal duration (15–17 days) were used for this study. Day 0 of the estrous cycle (standing estrus) was determined by using vasectomized rams.

**Experiment 1: Expression of eNOS protein in healthy and atretic follicles**

Ewes were treated twice daily with i.m. injections of saline or FSH-P (Schering Corp., Kenilworth, NY, USA) on days 13 (5 FSH units/injection, day 0 = estrus), 14 (4 FSH units/injection), and/or 15 (3 FSH units/injection) of the estrous cycle to induce follicular growth or atresia (Jablonka-Shariff et al. 1996). Ewes were randomly assigned to four treatment groups (n = 4 ewes/group): group 1 was treated with saline on days 13–15; group 2 was treated with FSH on day 13, and with saline on days 14 and 15; group 3 was treated with FSH on days 13 and 14, and with saline on day 15; and group 4 was treated with FSH on days 13–15. These treatments provided an animal model correspondingly as non-atretic healthy, early atretic, or advanced atretic. Healthy antral follicles were characterized as having a thick, continuous granulosa cell layer, and fewer than 10 pyknotic nuclei per cross-section. Early atretic follicles were characterized by thinning or loosening of the granulosa cell layer and by the presence of greater than 10 pyknotic nuclei per cross-section in the antrum. Advanced atretic follicles had a thinner granulosa cell layer than early atretic follicles and more than 10 pyknotic nuclei per cross-section. The experimental design, methodology, and the resulting follicular status and morphology have been previously described (Jablonka-Shariff et al. 1996).

**Experiment 2: Expression of eNOS protein and the mRNA for eNOS and GUCY1B3 in periovulatory follicles and corpora hemorrhagica**

Ewes were treated twice daily with i.m. injections of FSH-P (Sioux Biochemical, Sioux Center, IA, USA) on days 13 (5 units/injection) and 14 (4 units/injection), and both FSH-P (5 units/injection) and 600 IU hCG (Chorulon; Intervet, Milford, DE, USA) on the morning of day 15 after estrus. Ewes (n = 5/group) were assigned randomly for slaughter at 0, 2, 4, 8, 12, 24, and 48 h after hCG treatment. At slaughter, ovaries were collected and then washed using PBS containing 2% penicillin and streptomycin (Gibco). The ovary was placed into a 60 mm petri dish containing serum free Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 100 µg/ml heparin (Sigma). One or two pieces from each ovary containing preovulatory and/or postovulatory follicles were fixed in Carnoy’s solution. For the remaining follicles (>4 mm diameter), follicular fluid was aspirated and follicles were cut open. Granulosa cells were separated from the follicles using a siliconized Pasteur pipette and trituration in DMEM with heparin. Theca tissue was peeled from each follicle using a forceps. For each sheep, theca and granulosa tissues were pooled separately and snap frozen for mRNA extraction.

**Experiment 3: Expression of eNOS protein and the mRNA for eNOS and GUCY1B3 in the CL**

Ewes were treated twice daily with i.m. injections of FSH-P (Sioux Biochemical) as described for group 4 of experiment 1. Ovaries were collected on days 2, 4, 10, and 15 (n = 5 ewes/day) of the estrous cycle, which corresponds to the early (days 2 and 4), mid (day 10), and late (day 15) luteal stages of the estrous cycle. CL was dissected and separate portions were either fixed in Carnoy’s solution or snap-frozen for mRNA extraction.

**Immunohistochemistry**

Immunostaining of eNOS protein was performed using procedures described previously (Jablonka-Shariff et al. 1996, Redmer et al. 2001). Briefly, fixed tissues from...
et al. (2001). However, for the real-time reverse transcriptase (RT)-PCR analysis, the quality and quantity of total cellular RNA were determined via capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE, USA) as we have described previously (Redmer et al. 2005). In experiments 1 and 2, the morphological evaluations to categorize follicles as healthy or atretic were followed by evaluation of eNOS staining in 2–10 follicles per ovary. In experiments 1 and 2, in healthy small, medium, and large preovulatory follicles, eNOS protein was located exclusively in the blood vessels in the theca externa (TE) and in the theca interna (TI) layers (Fig. 1A–D). During the immediate postovulatory period, expression of eNOS protein was observed in the blood vessels of thecal-derived cells that appeared to be invading the granulosa layer (Fig. 1E). In early and advanced atretic follicles, expression of eNOS protein was reduced or absent in the intermediate postovulatory period in theca and granulosa cells for eNOS and GUCY1B3 mRNA was evaluated by regression analysis (SAS 1999) and the best-fit curves (greatest $R^2$) were selected.

### Results

Real-time RT-PCR

Expression of mRNA for eNOS and GUCY1B3 in granulosa and theca cells (experiment 2) were determined using quantitative real-time RT-PCR as described by Redmer et al. (2005). For the description of probe–primer sets for NOS3, and GUCY1B3, see Redmer et al. (2005).

RNase protection assay

Expression of mRNA for eNOS and GUCY1B3 in luteal tissues (experiment 3) was conducted using an RPA kit from Ambion (Austin, TX, USA) as described previously (Redmer et al. 1996). Quantification of RPA analysis was performed using autoradiography followed by densitometry (Redmer et al. 1996).

Statistical analysis

Data for eNOS protein staining and for eNOS and GUCY1B3 mRNA expressions were analyzed using the general linear model (GLM) procedure of SAS (1999) and Levene’s Test for Homogeneity of Variance (Levene 1960). When the $F$-test was significant, differences between specific means were further evaluated by using the Bonferroni’s $t$-test (SAS 1999). In addition, the pattern of change during the periovulatory period in theca and granulosa cells for eNOS and GUCY1B3 mRNA tended ($P<0.08$) to increase in granulosa cells at 12 and 24 h, and in theca cells at 48 h after hCG-treatment (Fig. 2). Expression of GUCY1B3 mRNA in granulosa or theca cells was not affected by hCG treatment (data not shown). Regression analysis demonstrated that eNOS mRNA expression in the thecal layer was described by a linear model ($y=1.448+0.078x$; $R^2=0.278$; $P<0.001$) and increased from 0 to 48 h after hCG-treatment.

### Isolation of RNA

Total cellular (tc)RNA was isolated from snap-frozen tissues using TriReagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s recommendations. The determination of quality and quantity of tcRNA used for RNase protection assay (RPA) was based on gel electrophoresis and spectrophotometry (Redmer et al. 1996). However, for the real-time reverse transcriptase (RT)-PCR analysis, the quality and quantity of total cellular RNA were determined via capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE, USA) as we have described previously (Redmer et al. 2005).
In experiment 3, eNOS protein was located in the blood vessels of luteal tissues (Fig. 3). On days 2, 4, and 10 of the estrous cycle, eNOS protein was detected in capillaries, and in small to large blood vessels in the parenchymal areas and connective tissue tracts of the CL. However, on day 15 of the estrous cycle, eNOS protein expression was primarily in the larger blood vessels of the parenchymal areas and connective tissue tracts (Fig. 3). The area of positive staining for eNOS in luteal tissues was the greatest \( (P<0.01) \) on day 4, less \( (P<0.01) \) on days 2 and 10, and was least \( (P<0.01) \) on day 15 of the estrous cycle (Fig. 4A).

Expression of eNOS mRNA in the CL was greater \( (P<0.05) \) on days 2 and 4 than on days 10 and 15 of the estrous cycle (Fig. 4B), and resembles the pattern of eNOS protein expression demonstrated by immunohistochemistry. By contrast, the expression of GUCY1B3 mRNA was unchanged in the CL across all days of the estrous cycle (Fig. 4B). Thus, the luteal expression of eNOS protein and mRNA were greatest, early in the estrous cycle when luteal vascular development also occurred.

Co-localization of eNOS and VEGF demonstrated that both were found in blood vessels of the CL throughout the estrous cycle; eNOS was detected in endothelial cells and VEGF in pericytes and smooth muscle cells (Fig. 5).

**Discussion**

We have shown that expression of eNOS changes throughout follicular growth and atresia, and during the growth, differentiation, and regression of the CL. Others have demonstrated that changes in eNOS expression are reflected by NO production (Zheng et al. 2000). Therefore, the expression of eNOS can be used as a marker of NO presence and/or production.

NO is one of several intraovarian mediators that have been shown to influence ovarian function, including follicular development and atresia, ovulation, steroidogenesis, oocyte quality, apoptosis, and luteal function (Rosselli et al. 1998, Dixit & Parviz 2001, Gregg 2003, Maul et al. 2003, Goud et al. 2005, Skarzynski et al. 2005). In addition, NO may positively regulate the expression of angiogenic factors, including VEGF and

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Figure 1 Immunohistochemical localization of eNOS in sections of ovarian follicles: (A) small, (B) medium, (C) large, (D) preovulatory, (E) postovulatory, and (F) atretic (F). The blackish color indicates positive eNOS staining. Note intensive staining (arrows) in blood vessels in the theca layer of healthy follicles ((A)–(E)), but no staining in the atretic follicle (F). Pycnotic nuclei (arrowheads) are visible in the atretic (F) but not in the healthy follicles. G, granulosa cell layer; TI, theca interna; TE, theca externa. Control sections were negative for eNOS staining (data not shown). Magnification using 40× objective.
the angiopoietins in the ovaries and other tissues (Reynolds et al. 2000, Cooke 2003, Fam et al. 2003). Since the follicles and CL function in a cyclic manner, and both are highly vascularized, the regulation of ovarian blood vessel development, maintenance, and function must be tightly regulated (Reynolds et al. 2000, 2002). NO, along with VEGF, the angiopoietins and several other factors, is recognized as one of the major angiogenic factors that regulate angiogenesis and vascular function (Reynolds et al. 2000, 2002, Grazul-Bilska et al. 2001, Fam et al. 2003).

In the present study, eNOS protein was immunolocalized exclusively within the blood vessels of the TE and TI in developing, preovulatory, and postovulatory healthy follicles. However, eNOS mRNA was detected in both theca and granulosa layers of ovine ovarian follicles. Thus, the pattern of eNOS protein expression followed the pattern of vascular development during folliculogenesis (Augustin 2001). However, in rodents, eNOS protein was also localized to oocytes, and theca, granulosa, and/or stromal cells (Nakamura et al. 1999, Zackrisson et al. 1996, Jablonka-Shariff & Olson 1998, Mitchell et al. 2004). This indicates that there are species-specific differences in the pattern of eNOS protein expression. During folliculogenesis, NO not only regulates angiogenesis and blood vessel function, but also is involved in the regulation of steroidogenesis by influencing estradiol and progesterone production by granulosa and theca cells in several species (Olson et al. 1996, Yamauchi et al. 1997, Basini et al. 1998, Rosselli et al. 1998, Maul et al. 2003). Therefore, it appears that NO plays a complex role in regulation of follicular development. Since we did not detect eNOS protein in granulosa cells, this indicates that the level of expression was very low and therefore, NO may not be involved in the regulation of granulosa cell function in sheep. However, this subject requires additional investigation.

The importance of the follicular vasculature for maintaining follicular health has been emphasized in several studies (Hirshfield 1991, Mattioli et al. 2001, Zeleznik 2001, Jiang et al. 2003). In addition, reduced follicular vascularity is one of the earliest signs of atresia (Zeleznik & Benyo 1994) marked by a smaller vascular network and increased apoptosis in thecal capillaries (Mattioli et al. 2001, Watson & Al-Zi’abi 2002, Jiang et al. 2003). Therefore, increased thecal vascularity may be a primary determinant of follicular dominance and, conversely reduced thecal vascularity, a marker of follicular atresia (Redmer & Reynolds 1996, Reynolds...
et al. 2000, Augustin 2001). The greater expression of eNOS seen in blood vessels of healthy follicles compared with atretic follicles in this study also supports a concept that NO has a role in maintaining or developing vascularization during folliculogenesis, follicular growth, and/or selection of follicles for ovulation. In fact, it has been hypothesized that healthy antral follicles require angiogenic factors and gonadotropins to support follicular growth and prevent atresia in mammalian species, including sheep (Jablonka-Shariff et al. 1994, 1996, Zeleznik 2001).

Around the time of ovulation, structural and functional changes occur in granulosa and theca layers, which include cellular proliferation, remodeling, and vascularization leading to transformation of the ruptured follicle into a CL (Redmer et al. 2001, Acosta & Miyamoto 2004). After ovulation, microvascular growth becomes very extensive and the thecal vasculature begins to invade the avascular granulosa layer to form a new blood vessel network (Redmer & Reynolds 1996, Reynolds et al. 2000, 2002). The growth of new capillaries during luteal angiogenesis follows a cascade of events that include changes in the basement membrane, and the migration and proliferation of endothelial cells and pericytes (Redmer & Reynolds 1996, Redmer et al. 2001).

In the present study, increased eNOS protein expression was detected in the dense blood vessel network in the theca layer along the basement membrane during the immediate preovulatory period, and expression of eNOS mRNA in the theca layer increased as well. Similar to our findings, increased expression of eNOS protein was observed in ovarian follicles after hCG treatment in rats (Jablonka-Shariff & Olson 1997, Nakamura et al. 1999). The enhanced eNOS expression accompanied a denser capillary network, which likely serves as a source of NO production in preovulatory follicles. During the preovulatory period, NO may increase both angiogenesis and blood flow to allow for increased supply of regulatory factors necessary for ovulatory processes (Acosta & Miyamoto 2004).

The increased expression of eNOS during immediate peri- and postovulatory periods observed in this and other studies suggest that NO is also important during the transition from a mature follicle into the CL. In mice,
Several studies have indicated a role for NO in luteolysis, hours after ovulation (Redmer et al. 1996, Yamauchi et al. 1997, Rosselli et al. 1998, Gregg 2003, Mitchell et al. 2004). Based on these observations, we hypothesize that NO regulates ovulatory processes directly by affecting blood vessel function and indirectly by affecting regulatory factors involved in ovulation.

In the present study, eNOS was highly expressed in the corpora hemorrhagica and early CL, and was localized in the invading thecal-derived cells that were forming new blood vessels. However, eNOS expression decreased in the differentiated and regressing CL, and was localized to capillaries, as well as to small and large blood vessels. Similar to our results, eNOS protein was found in the blood vessels of the CL in rats and cows (Olson et al. 1996, Zackerison et al. 1996, Skarzynski et al. 2005). In sheep and rabbits, there was increased activity of NOS in the early CL compared with the later, differentiated CL, which indicated that eNOS expression and activity followed a similar pattern (Boitti et al. 2002, Al-Gubory et al. 2005). Since the expression of eNOS in the CL changes during the estrous cycle, we hypothesize that a regulatory factor(s), such as VEGF and/or others, affects eNOS expression during specific stages of the luteal phase.

Our hypothesis is supported by observations in this study that eNOS and VEGF are both expressed in the blood vessels, and the expressions of eNOS and VEGF have a similar pattern, with greatest expression during the early luteal phase and decreasing expression during the mid- and late luteal phases (Redmer et al. 1996). VEGF, like eNOS, is expressed in thecal-derived cells within hours after ovulation (Redmer et al. 2001). Increased eNOS expression at early luteal development may be associated with the proliferation of endothelial cells and pericytes that produce eNOS and VEGF respectively (Redmer et al. 2001, Reynolds et al. 2002). Expression of eNOS is very likely enhanced by VEGF, since VEGF has been demonstrated to upregulate eNOS expression in several tissues (Cooke 2003, Duda et al. 2004). Observations from this and other studies further emphasize the relationship between eNOS and VEGF (Reynolds et al. 2000, 2002, Redmer et al. 2001, Yang et al. 2001, Duda et al. 2004, Beckman et al. 2006).

In the present experiment, eNOS protein expression decreased in the regressing CL (represented by day 15) compared with the early or mid-cycle CL. Decreased eNOS expression may be associated with either a reduced number of endothelial cells or pericytes, a reduced vasculature during luteal regression, and/or with a decreased expression of regulatory factors (Redmer et al. 1996, 2001, Vonnahme et al. 2006). Several studies have indicated a role for NO in luteolysis, because NO inhibited progesterone secretion in cows and rats (Motta & Gimeno 1997, Skarzynski et al. 2005). It has been suggested that during luteolysis NO may interact with such luteolytic factors as PGF2α or endothelin 1 (Tognetti et al. 2003, Boitti et al. 2005). However, it has been demonstrated that NO can reverse PGF2α-induced inhibition of progesterone secretion by rat and sheep CL, which suggests an anti-luteolytic role for NO (Dong et al. 1999, Weems et al. 2005). The mechanism for an anti-luteolytic and/or luteotropic action of NO has not been elucidated, therefore, this subject requires further investigation.

In our study, eNOS expression was reduced but still present in the CL during the late luteal phase of the estrous cycle. Therefore, we hypothesize that a decreased level of eNOS expression may be required for luteolytic processes, whereas a greater expression of eNOS may be necessary for angiogenesis and maintenance of blood vessel function at the earlier stages of luteal development. In fact, a dual role of NO in regulation of luteal function, being luteotropic during the mid-luteal stage and luteolytic during the late luteal phase, has been suggested by Motta et al. (2001).

We have demonstrated that GUCY1B3 mRNA is expressed in granulosa, thecal, and luteal tissues, and that the level of expression did not change during the estrous cycle. An unchanged GUCY1B3 protein expression during the estrous cycle has also been reported in the ovaries of rats (Shi et al. 2004). In addition, GUCY1B3 protein was immunolocalized in the granulosa cells of developing follicles, thecal tissues, luteal cells, and ovarian blood vessels in rats (Shi et al. 2004). Since GUCY1B3 increases cGMP levels, it has been suggested that GUCY1B3 may affect steroidogenesis in ovarian cells (Tafoya et al. 2004). However, to pinpoint a specific role for the NO receptor, GUCY1B3 in ovarian function, additional studies are needed.

In summary, this comprehensive report of ovarian eNOS expression demonstrates that eNOS protein and mRNA expression changes substantially during follicular and luteal growth, differentiation, and regression. These data indicate that NO, probably in combination with VEGF, plays a role in regulation of follicular and luteal angiogenesis and maintenance of vascular function. Based on our data and from several other studies, it seems reasonable to conclude that inappropriate follicular and luteal expression of eNOS may contribute to ovarian vascular defects and dysfunction. This concept has been supported by an additional observation that NO suppression by inhibitor L-NAME, caused reduction of size of follicular cysts, sustained testosterone levels, and maintained hormonal blood follicle barrier reactivity in cystic follicles in mice with ovarian cysts induced by hCG-treatment (Nemade et al. 2002).
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