Hormonal and Spatial Regulation of Nitric Oxide Synthases (NOS) (Neuronal NOS, Inducible NOS, and Endothelial NOS) in the Oviducts

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Nitric oxide (NO) is a free radical gas synthesized from L-arginine by NO synthase (NOS), a family of three different enzyme isoforms encoded by three distinct genes (1). These isoforms are neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (2–4). Both nNOS and eNOS are present in many cell types and activated by calcium and calmodulin. Although these two enzymes were initially considered as constitutively expressed, it is now established that the expression of nNOS and eNOS can be regulated at the transcriptional level under various conditions (5). The third isoform, iNOS, is a calcium-independent enzyme known to be expressed in macrophages, smooth muscle cells, monocytes, endothelial cells, neurons, and hepatocytes (6, 7). In these cells, iNOS expression can be induced by immunostimulatory cytokines, oxidative stress and bacterial products (8, 9).

NO freely diffuses through the membranes and acts as an intracellular and extracellular biological messenger in a variety of physiological processes such as host defense, neuronal communication, and regulation of vascular tone (10–12). In addition to this wide range of physiological actions, NO has also been implicated in the regulation of mammalian reproduction (13–16). Moreover, male mice deficient in neuronal NOS are infertile and ejaculatory abnormalities were observed in mice lacking the gene for eNOS (15, 17). NO also mediates many female reproductive functions in mammals and appears to be a major paracrine regulator of various female reproductive processes such as gonadotrophin secretion, ovulation, corpus luteum regression, implantation, embryonic development, uterine contractility, cervical ripening, and parturition (14, 18–20). All three NOS isoforms have been detected alone or in combination in the vaginal, cervical, placental, and uterine tissues of many species (21–24). eNOS knockout mice have reduced numbers of ovulated oocytes after superovulation and abnormal estrous cycle length (25, 26). Reduced fertility is also observed in knockout female mice lacking both eNOS and iNOS isoforms (27). Additionally, recent experiments provide convincing evidence that nitric oxide can affect all the reproductive events that take place in the oviduct in vivo (28–31).

The mammalian oviducts are highly specialized organs that provide the optimal conditions for the gamete maturation, fertilization, and early embryo development (32, 33). Within the oviducts the male and female gametes are transported to the site of fertilization at the isthmic-ampullary junction, followed by embryo transfer to the uterus (34, 35). If one of these oviducal functions is altered, reproductive success is seriously compromised (36, 37). The importance of NOS isoforms and their roles in oviducts are differentially distributed in cells along the oviduct. Interestingly, our results showed that estradiol selectively up-regulates iNOS expression in the oviduct during the periovulatory period corresponding to the window of ovulation, oocyte transport, and fertilization. The resulting NO production by this high-output NOS may be of crucial importance for reproductive events that occur in the oviduct. This study provided the first demonstration that NO production is hormonally regulated in the mammalian oviducts in vivo. Our results suggest that neuronal NOS, iNOS, and endothelial NOS contribute to oviducal functions in a timely and site-specific manner. (Endocrinology 147: 5600–5610, 2006)
NO has been linked to essential oviduct functions. NO was first implicated in oocyte maturation because severe maturation defects were observed in oocytes recovered from the oviducts of rats treated with NOS inhibitors (30). Interestingly, it was reported that low concentration of NO was beneficial to the maintenance of sperm viability and the induction of sperm capacitation in several species (13, 38). Moreover, several studies revealed that the binding of sperm to the zona pellucida and gametes fusion are promoted by low levels of NO and blocked by NOS inhibitors as well as high concentrations of NO-releasing compounds (29, 39–41). Critical concentrations of NO are also required for normal preimplantation embryo development. Deviations from these concentrations lead to developmental arrest of the embryo (31, 42, 43). Other studies indicated that NO is involved in the regulation of oviductal smooth muscle contractions (44–46). It was found that NO plays a role as a relaxing agent in mammalian oviduct and NO inhibition increases tubal motility that results in accelerated ovum transport (47, 48). Thus, it appears that NO production must be tightly regulated in the oviduct to favor the proper condition for gametes maturation, fertilization, early embryonic development, and embryo transfer to the uterus. To date, few studies have reported NOS activity as well as the presence of NOS enzymes in mammalian oviducts (49–51). However, almost nothing is known on NOS expression and distribution in the different segments of the mammalian oviduct. Furthermore, the factors controlling the expression of the different NOS isoforms in the oviduct are not clearly established.

We determined the regulation and distribution of nNOS, iNOS, and eNOS in the oviduct segments during the estrous cycle of the cow. We found a specific expression pattern for each NOS isoforms in the oviduct throughout the estrous cycle. Importantly, our results revealed that estradiol selectively up-regulates iNOS expression in the oviduct in vivo. We propose that this specific regulation of the inducible high-output NOS could play important roles in reproductive events that naturally occur in the oviducts during the periovulatory stage.

**Materials and Methods**

**Tissue samples**

Oviducts as well as the other bovine tissues analyzed in this study were transported on ice to the laboratory within 4 h of the animal being killed. Animals that showed anomalies of the genital tract were rejected after examination by a veterinarian. The stage of the estrous cycle was determined in the oviduct to favor the proper condition for gametes maturation, fertilization, early embryonic development, and embryo transfer to the uterus. To date, few studies have reported NOS activity as well as the presence of NOS enzymes in mammalian oviducts (49–51). However, almost nothing is known on NOS expression and distribution in the different segments of the mammalian oviduct. Furthermore, the factors controlling the expression of the different NOS isoforms in the oviduct are not clearly established.

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**Treatments**

All procedures were performed in accordance to the guidelines of the Canadian Council on Animal Care and were reviewed and approved by the Nova Scotia Agricultural College Animal Care and Committee. Six healthy, sexually mature mixed mixed-graded beef heifers (1.5–3 yr of age; 520 ± 31 kg body weight) were randomly assigned to control (BSA, n = 3) or 17β-estradiol (n = 3) intratracheal infusions treatments as previously described by Kimmins et al. (53). Briefly, animals were treated midcycle with Estrumate (500 mg cloprostenol; Schering Canada Inc., PointeClaire, Quebec, Canada) to synchronize estrus. In the morning of d 14 after estrus, each heifer received intratracheal infusions in both horns of BSA (0.9% saline) or the estradiol (117 mg, 2x 58.5 mg/d horn) using 12-gauge Foley catheters (Agtech, Manhattan, KS). At the time of treatment, the horns were gently massaged to ensure distribution to the oviducts. Treatments were delivered five times at 12-h intervals. The animals were slaughtered 7 h after the last treatment on d 16 of the estrous cycle and the oviducts were collected. NO has been linked to essential oviduct functions. NO was first implicated in oocyte maturation because severe maturation defects were observed in oocytes recovered from the oviducts of rats treated with NOS inhibitors (30). Interestingly, it was reported that low concentration of NO was beneficial to the maintenance of sperm viability and the induction of sperm capacitation in several species (13, 38). Moreover, several studies revealed that the binding of sperm to the zona pellucida and gametes fusion are promoted by low levels of NO and blocked by NOS inhibitors as well as high concentrations of NO-releasing compounds (29, 39–41). Critical concentrations of NO are also required for normal preimplantation embryo development. Deviations from these concentrations lead to developmental arrest of the embryo (31, 42, 43). Other studies indicated that NO is involved in the regulation of oviductal smooth muscle contractions (44–46). It was found that NO plays a role as a relaxing agent in mammalian oviduct and NO inhibition increases tubal motility that results in accelerated ovum transport (47, 48). Thus, it appears that NO production must be tightly regulated in the oviduct to favor the proper condition for gametes maturation, fertilization, early embryonic development, and embryo transfer to the uterus. To date, few studies have reported NOS activity as well as the presence of NOS enzymes in mammalian oviducts (49–51). However, almost nothing is known on NOS expression and distribution in the different segments of the mammalian oviduct. Furthermore, the factors controlling the expression of the different NOS isoforms in the oviduct are not clearly established.

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**Measurement of 17β-estradiol levels in serum**

Serum estradiol levels were measured by a gas chromatographic mass spectrometric method developed to measure steroid hormone levels in rat and monkey serum (54). Briefly, 17β-estradiol was extracted from serum by liquid-liquid and solid-phase extraction. Derivatization reactions were performed to improve chromatographic and detection responses of the steroids. Unconjugated estradiol was quantified by means of a sensitive gas chromatographic/mass spectrometric method, using chemical ionization.

**Preparation of RNA and cDNA**

The RNA was extracted using TRIzol as described in the manufacturer’s instructions (Invitrogen, Burlington, Ontario, Canada). Four micrograms of total RNA were reverse (transcribed with random hexamer primers and the Superscript II reverse transcriptase (Invitrogen). The first-strand cDNA was diluted 20 times in sterile water and used as the template in the quantitative RT-PCR mixture. Ipsilateral and contralateral oviducts were pooled together before RNA extraction to prepare whole oviduct samples.

**Quantitative RT-PCR**

Sets of specific primers were designed based on known bovine sequences to amplify specific products for nNOS (forward: 5′-gcattcttggtgcgtcttccttaa-3′; reverse: 5′-gctgggcaacagggatgtctg-3′), iNOS (forward: 5′-agccagggagttgctcctc-3′; reverse: 5′-ctgctagcttgagttctg-3′), and eNOS (forward: 5′-ctctgtgacacgcaaga-3′; reverse: 5′-cagatatccagctggctg-3′). Classical PCRs were performed first to confirm the specificity of primers. As an internal control, 18S rRNA was amplified using specific primers (forward: 5′-gttataacctgctaacacct-3′; reverse: 5′-tcattctggaggtggagc-3′). The expected PCR products were visualized by agarose gel electrophoresis, eluted, and sequenced (Center of Genomic, Centre Hospitalier de l’Université Laval, Quebec, Canada). These specific NOS PCR products were serially diluted from 500 pg to 5 fg and used as templates in quantitative RT-PCR to establish the standard curves. The quantitative RT-PCR were carried out in a LightCycler (Roche Diagnostics, Laval, Quebec, Canada). Reactions were performed in a 20-μl reaction mixture containing 0.5 μl diluted cDNA or PCR product, 0.58 μM of each primer, 3 mM of MgCl2, 2 μl of FastStart Master SYBRGreen I mix (Roche Diagnostics), and PCR-grade water up to the final volume. The RT-PCRs were performed as follows: denaturation at 95 C for 10 min followed by 45 cycles of amplification (95 C for 0 sec, annealing temperature for 5 sec, and 72 C for 20 sec) with single acquisition of fluorescence at the end of extension step. The annealing temperatures for each gene were: nNOS (66 C); iNOS (63 C); eNOS (67 C); and 18S (58 C). After amplification, the samples were slowly heated at 0.1 C/sec from 60 to 95 C with continuous reading of fluorescence to obtain a melting curve. The specificity of each amplicon was then determined by using the melting curve analysis program of the LightCycler software. The amplicon of each NOS and 18S showed only one peak in the analysis. The specificity of the amplified products was confirmed by agarose gel electrophoresis. The quantification analysis of
the data were performed by using the LightCycler analysis software as previously described (55). Relative gene expression was expressed as a ratio of target gene concentration to 18S rRNA. All total RNA samples were reverse transcribed twice. Each cDNA was quantified in duplicate and the average value of each sample was used for quantification.

Western analysis

Protein samples (50 μg) from isthmus, isthmic-ampullary junction and ampulla sections were boiled in Laemmli’s sample buffer containing 5% (vol/vol) of 2-mercaptoethanol for 10 min and loaded (50 μg of protein per track) on 10% polyacrylamide gels, blotted, and processed as previously described using rabbit polyclonal antirat nNOS (Abcam, Cambridge, MA), rabbit polyclonal antitumour iNOS (Cedarlane, Hornby, Ontario, Canada), mouse monoclonal antibovine eNOS (Stressgen, San Diego, CA), and polyclonal rabbit antinitrotyrosine (Upstate Biotechnology, Lake Placid, NY) (56). All the antibodies for NOS isoforms were known to cross-react with the corresponding bovine protein. Isthmus, isthmic-ampullary junction and ampulla sections from five different specimens were analyzed for NOS expression at each phase of the estrous cycle.

Immunohistochemistry

Oviduct segments were fixed overnight in 4% paraformaldehyde, embedded in optimum cutting temperature medium, frozen in liquid nitrogen, and stored at −86°C (Canemco, St. Laurent, Quebec, Canada). Cryosections of 8 μm were prepared from frozen oviductal tissues using a cryotome (Shandon, Pittsburgh, PA). Immunohistochemical detection of each NOS isoform was performed using Vectastain Elite ABC kit according to the manufacturer’s protocols (Vector Laboratories Inc., Burlingame, CA). Endogenous peroxidase activity was first inactivated with 3% H2O2 (vol/vol) in methanol for 30 min. Nonspecific binding sites were then blocked with 10% goat serum (Sigma-Aldrich, Oakville, Ontario, Canada) in PBS for 1 h at room temperature. Incubation with the primary NOS antibody was performed overnight at 4°C. Antibodies used were rabbit polyclonal antirat nNOS (Abcam), rabbit polyclonal antitumour iNOS (Cedarlane), mouse monoclonal antibovine eNOS (Calsbiochem, San Diego, CA), and polyclonal rabbit antinitrotyrosine (Upstate). Each antibody was used at a dilution of 1:400 (vol/vol). Corresponding nonspecific IgGs were used as negative control and processed in parallel. The oviduct cryosections were further washed with PBS and incubated at room temperature with the second antibody (goat antirabbit or antimouse IgG biotinylated, 1:200) for 1 h and ABC elite reagent with 3% H2O2 (vol/vol). Nonspecific binding sites were then blocked with 10% goat serum (Sigma-Aldrich, Oakville, Ontario, Canada) from isthmus, isthmic-ampullary junction and ampulla sections. Immunostaining was revealed using 3-amino-9-ethylcarbazole, and sections were counterstained with Mayer’s hematoxylin (Sigma-Aldrich).

Three separate experiments were performed on five specimens using eight cryosections per specimen. Because no differences in NOS expression pattern were observed between the different stages of the estrous cycle, we showed only specimens from postovulatory stage. Images were acquired in color directly from the stained tissue with a Axioskop 2 Plus microscope (Zeiss, Toronto, Ontario, Canada) linked to a digital camera using Spot software (Diagnostic Instruments, Sterling Heights, MI). The isthmus and ampulla sections from estradiol-treated and untreated animals were fixed in 4% paraformaldehyde and embedded in paraffin. The immunohistochemical detection of iNOS was performed as previously described (57). The antibody dilution was 1:200 (vol/vol). Relative quantification of the specific staining was performed by densitometry analysis using Image Pro software (Carsen Medical Scientific, Markham, Ontario, Canada) as previously described (58).

Statistical analysis

Values shown in the text and tables are mean ± SEM. All data were normally distributed and passed equal variance testing. Model variables included oviduct section (isthmus, isthmic-ampullary junction, and ampulla), oviduct side (ipsilateral vs. contralateral to the corpus luteum), and stage of the estrous cycle (d 0–3, 10–12, 15–17, and 18–20). Main effects of each variables and interactions between these variables were determined. The experiments were analyzed with the general linear model of SPSS 10.0 for Windows (SPSS Inc., Chicago, IL). Multiple means were compared by ANOVA, and when a significant effect was obtained, the difference between means was determined by Duncan multiple range test. P < 0.05 was considered statistically significant.

Results

The three NOS isoforms (nNOS, iNOS, and eNOS) are expressed in the bovine oviduct

We first used quantitative RT-PCR to detect and measure NOS mRNA expression in the bovine oviduct. The relative abundance of each NOS mRNA in the oviduct in comparison with other tissues is shown in Fig. 1. We found that all NOS isoforms (nNOS, iNOS, and eNOS) were expressed in the oviduct. It was known that nNOS was expressed at a high level in the neuronal connections, and our results revealed that nNOS mRNA expression in the oviduct is similar to the levels found in the liver and the kidney and was five times lower than in the brain (Fig. 1A). Surprisingly, we observed that the inducible NOS was strongly expressed in the oviduct. Indeed, iNOS expression in the oviduct was significantly higher than in the majority of other tissues investigated and attained the levels of expression measured in the heart (Fig. 1B). As expected, our analysis also revealed that eNOS mRNA was present in the oviducts. Of note, eNOS is the only NOS isoform found expressed in the oviduct and uterus to the same extent (Fig. 1C).

The NOS isoforms are differentially expressed throughout the estrous cycle and along the oviduct

To analyze the effect of hormonal status on the expression of the NOS, we first measured NOS mRNA expression at the postovulatory (d 0–3), midluteal (d 10–12), late luteal (d 15–17), and follicular (d 18–20) stages of the estrous cycle by quantitative RT-PCR. Interestingly, we found that all NOS were differentially expressed in the whole oviduct during the estrous cycle (Fig. 2). Indeed, a significant impact of the estrous cycle on nNOS expression was observed. We demonstrated that nNOS expression was highest during the periovulatory period (d 0–3 and 18–20; Fig. 2A). The highest expression level for iNOS mRNA was observed after ovulation, between d 0 and 3 of the estrous cycle (Fig. 2C). However, in contrast to nNOS, iNOS expression decreased significantly during the luteal phases and remained low at the follicular stages. Furthermore, our results revealed a different expression pattern for eNOS throughout the estrous cycle (Fig. 2E). Indeed, eNOS expression was maximal at the end of the estrous cycle and decreased subsequently to reach is minimal level during the luteal phases.

To further investigate the regional mRNA and protein expression of the three NOS along the oviduct, three equidistant sections of the oviduct were examined at the same four stages of the estrous cycle. Quantitative RT-PCR analysis above revealed that nNOS was highly expressed in the isthmus region of the oviduct at all times of the estrous cycle, but no significant variations were measured (Fig. 2B). However, we observed that the protein level of nNOS was higher in the isthmus during the luteal phase and in the ampulla section during the periovulatory period of the cycle (Fig. 3). In contrast to nNOS, we found that iNOS expression was higher in the ampulla shortly after the ovulation (Fig. 2D).
During the others stages of the estrous cycle, iNOS mRNA expression was significantly lower in the isthmus region than in the isthmic-ampullary junction and the ampulla segment (Fig. 2D). In accordance with mRNA results, iNOS protein was highly expressed in the ampulla section during the postovulatory stage of the estrous cycle (Fig. 3). In contrast to iNOS, the expression of eNOS mRNA was 2-fold higher in the isthmus than the ampulla sections. This was observed only during the periovulatory period (Fig. 2F). Furthermore, we found that eNOS protein was mostly expressed in the ampulla during the middle and end of the estrous cycle, which contrast to mRNA expression (Fig. 3).

Cellular distribution of nNOS, iNOS, and eNOS proteins along the oviduct

We localized the cellular expression of the three NOS isoforms along the bovine oviduct using immunohistochemistry. Our analysis revealed specific expression patterns for each NOS protein in the isthmus, the isthmic-ampullary junction, and the ampulla segment of the oviduct. The cellular distribution of nNOS, iNOS, and eNOS in isthmus, isthmic-ampullary junction, and ampulla is consistent throughout the estrous cycle (data not shown). Neuronal NOS protein was observed in oviductal epithelium, lamina propria and smooth muscle cells of all sections (Fig. 4, A–C). In the isthmus, strong staining intensity for nNOS is observed in the thick smooth muscle layer and epithelial cells, whereas the signal was weaker in the lamina propria (Fig. 4A). Although similar nNOS distribution was found in the ampulla, nNOS expression appeared to be more homogenous in the isthmic-ampullary junction of all cryosections investigated (Fig. 4, B and C).

Inducible NOS was mainly detected in the smooth muscle and epithelial cells of the ampulla sections (Fig. 4F). Indeed, only weak iNOS-specific staining was observed in the smooth muscle cells of the isthmic-ampullary junction as well as in the lamina propria of the isthmus (Fig. 4, D and E). Interestingly, we also found that iNOS was principally localized on the luminal surface of the epithelial cells in the ampulla (Fig. 4F).

In Fig. 4, we showed that eNOS cellular distribution varied between the oviduct segments. In the isthmus, eNOS was detected in the epithelial cells as well as the endothelium of blood vessels present in the lamina propria and serosa (Fig. 4G). eNOS was expressed in all oviduct cell types in the isthmic-ampullary junction and ampulla (Fig. 4, H and I).

To gain further insight into NOS functions in the different oviduct segments, nitration of tyrosine residues in oviduct proteins, a marker of NOS biological activity, was evaluated by immunohistochemical staining using antinitrotyrosine antibody. In the isthmus, strong protein nitrotyrosination signal was detected in the smooth muscle layer as well as in the epithelium, whereas weaker signal was seen in the lamina propria (Fig. 4J). In contrast, homogenous staining was observed in all oviduct tissues in the isthmic-ampullary junction (Fig. 4K). In the ampulla segment, specific protein nitrotyrosination staining was also found in all cellular types, but we observed that the epithelial staining was restricted to the luminal surface of the cells like iNOS expression (Fig. 4, L and F). No significant signal was detected with the non-specific IgG in the control sections (Fig. 4, M–O).
Expression of nNOS, iNOS, and eNOS in the ipsilateral and contralateral oviducts during the estrous cycle

To better characterize NOS regulation during the estrous cycle, we analyzed their expression in oviducts ipsilateral and contralateral to the cycling ovary. No significant variation of nNOS and eNOS expression was found between the ipsilateral and contralateral oviducts sections during the estrous cycle (Fig. 5, A and C). However, we observed that in the isthmus section, iNOS expression was significantly higher in the oviduct proximal to the dominant follicle during the follicular phase (Fig. 5B). Interestingly, iNOS expression was not modulated between the ipsilaterial and contralateral oviducts at the isthmic-ampullary junction and ampulla levels during the estrous cycle (Fig. 5B).

Fig. 2. NOS mRNA expression in the bovine oviduct throughout the estrous cycle. The mRNA levels were measured by real-time quantitative PCR. The 18S rRNA was used as an internal standard and results are expressed as a ratio of nNOS to 18S. A, C, and E, Expression of nNOS, iNOS, and eNOS in the whole oviduct at the postovulatory (d 0–3), midluteal (d 10–12), late luteal (d 15–17), and follicular (d 18–20) stages of the estrous cycle. B, D, and F, nNOS, iNOS, and eNOS expression in the isthmus ( ), isthmic-ampullary junction ( ), and ampulla ( ) segments of the oviduct at the four stages of the estrous cycle. Data are means ± SEM of five animals. Means with different designations (*, †) are significantly (P < 0.05) different from each other (Duncan’s test).
Effects of 17β-estradiol on NOS expression in the oviducts in vivo

We previously demonstrated that 17β-estradiol modulates the expression of certain antioxidant genes in the oviduct in vivo (55). Because we found in this study that each NOS transcripts are mostly expressed during the periovulatory period estrous cycle, we decided to test the hypothesis that estradiol regulates NOS expression in the bovine oviduct. Thus, heifers were treated with intrauterine infusions of 17β-estradiol or saline control, and we analyzed the mRNA expression levels of nNOS, iNOS, and eNOS in the whole oviduct. The 17β-estradiol levels for saline control animals were 2.46 ± 0.65 pg/ml of plasma. The treatment with estradiol caused a significant increase in circulating levels to 7.94 ± 2.06 pg/ml (mean ± SEM, P < 0.05, bilateral t test, n = 3). Our analysis revealed that 17β-estradiol specifically up-regulates iNOS expression in the oviduct. Indeed, iNOS expression was 2-fold higher in the oviducts of cows treated with uterine infusions of 17β-estradiol, whereas no variations were observed for nNOS and eNOS (Fig. 6, A–C). Interestingly, immunohistochemistry analysis also revealed that iNOS protein was up-regulated by estradiol in the isthmus and ampulla sections of oviducts from treated animals (Fig. 7, A–D). Moreover, relative quantification of iNOS-specific staining by densitometry analysis clearly indicated that iNOS protein expression was induced by about 3-fold in both isthmus and ampulla segments.

Discussion

Several in vitro and in vivo studies showed that nitric oxide is implicated in all the reproductive events that naturally occur in the mammalian oviduct (28, 31, 48, 59). Here we demonstrated that the three NOS were expressed in this specific organ of the female reproductive tract. Importantly, we found that iNOS mRNA was strongly expressed in the oviduct in comparison with other bovine tissues. Moreover, we observed that iNOS expression in the oviduct was significantly higher than in the uterus. Because it has been proposed that iNOS was implicated in crucial uterine functions in mammals, the latter observation also suggest an important role for iNOS in oviduct functions (18, 60).

The mammalian oviduct is a steroid-responsive organ, and it was shown that the expression of specific genes was modulated in the oviduct throughout the estrous cycle (36, 56, 61). Because it is now recognized that the level of NO produced by NOS, especially iNOS, could be regulated at the transcription level, we quantified the expression of each NOS isoform at different stages of the estrous cycle (5, 9). Consistent with transcriptional regulation, we observed a relationship between the mRNA expression measured by quantitative RT-PCR and the levels of proteins detected by immunoblots and immunohistochemistry along the oviduct throughout the estrous cycle, especially for iNOS. However, some differences between mRNA and proteins levels during the estrous cycle were observed for nNOS and eNOS and can probably be attributed to posttranscriptional regulation (62, 63). Of note, our RT-PCR analysis did not discriminate between the numerous spliced transcripts of the exon 1 and referred only to nNOS mRNA transcripts that contain exon 2, which is known to be deleted in two nNOS alternatively spliced forms (64). It might be of further interest to analyze the expression of these variants in the oviduct to ascribe specific functions to particular nNOS isoforms.

Strikingly, we found that all NOS were highly expressed during the periovulatory period. Indeed, the mRNA expression of nNOS and iNOS is maximal shortly after ovulation, whereas the highest eNOS expression level was measured at the end of the estrous cycle. This discovery is of high importance because this phase of the estrous cycle was characterized by an increase in oviductal muscular and secretory activity, two physiological processes that can be influenced by NO. The volume of fluid in the lumen of the mammalian oviduct is highest around the time of estrus and is formed in part by selective transudation from the blood (65). Indeed, the increasing accumulation of luminal fluid is closely related to the phase of dilatation of the neighboring blood vessels. Therefore, because NO was known to be a strong vasodilator, it is reasonable to believe that the high expression of NOS measured during the periovulatory period must be important to the formation of oviductal fluid. Moreover, the vasodilatation of blood and lymphatic vessels at the time of estrus also induces edematous conditions in the isthmic mucosa and reduces lumen diameter (66, 67). These actions are essential to maintain the direction of fluid flow and restrict sperm access to the oviduct. Thus, our data suggest that eNOS, which was mainly detected in the endothelium of the lamina propria blood vessels around estrus, may contribute to edema formation.

Contraction and relaxation of smooth muscle cells in the oviduct are the major factors involved in promoting and regulating the transport of gametes and embryo during the periovulatory period (35). NO has proved to be a mediator of smooth muscle relaxation in various tissues, and in vitro studies using organ baths have revealed that NO is involved in oviductal contractility (45, 46). Additionally, it was shown that NO inhibition increases oviductal motility that results in accelerated oocyte and embryo transport in vivo in rats (48). NO can modulate oviductal contractility by either inhibiting cyclooxygenase enzymes and prostanooids synthesis or activating cyclic GMP-dependent pathway (28, 68). Our current observations indicate that NOS are highly expressed at the appropriate time of the estrous cycle in the smooth muscle.
to modulate oviductal contractility in vivo. Interestingly, we reported that nNOS is the only isoforms expressed in the thick circular smooth muscle layer of the isthmus regions, which is known to be a very richly innervated region with high densities of adrenergic nerve terminals (69). Moreover, extreme constriction of this muscular region was observed during the follicular stage of the estrous cycle to control sperm transport to the site of fertilization at the isthmic-ampullary junction (35). In the postovulatory phase, this constriction decreases progressively to allow the passage of the embryo to the uterus. This latter observation is in concordance with our results showing that nNOS expression increases gradually from the follicular to postovulatory stage of the estrous cycle and the strong signal of protein nitrotyrosination detected in smooth muscle layer. This strongly suggests that nNOS is the NOS isoform that produces NO in the smooth muscle cells of the isthmus to regulate the smooth muscle relaxation as well as the passage of the embryo to the implantation site in the uterus.

The oviduct is composed of distinct segments of characteristic morphology, reflecting their different functional roles. In the present study, we clearly demonstrated that eNOS and iNOS were differentially expressed along the oviduct, whereas nNOS was equally expressed in all segments. The isthmus segment of the oviduct has been described as a sperm reservoir in several mammalian species, and we found that eNOS, nNOS, and iNOS are all expressed in the epithelial cells of this specialized region (70). We also observed a high level of protein nitrotyrosination in the epithelium of the isthmus.

These findings are important because it is well recognized that sperm bind to the isthmic epithelium and that NO production has beneficial effects on the maintenance of sperm viability and reduces sperm motility (13, 71). Therefore, we propose that exogenous NO production by the NOS isoforms in the reservoir during the follicular and postovulatory stages may contribute to maintain sperm in the appropriate physiological state for fertilizing oocytes. Moreover, NO is involved in events leading to fertilization in the oviduct such as sperm hyperactivation, capacitation, acrosome reaction,
and binding of sperm to zona pellucida (72, 73). Thus, NOS expression at the fertilization site in the isthmic-ampullary junction may be important to promote the fusion of gametes.

We also demonstrated that iNOS was highly expressed in the ampulla region during the periovulatory period. We found that iNOS was located at the luminal surface of the epithelial cells shortly after ovulation. Interestingly, we also showed that this specific region of the epithelial cells is characterized by a high level of protein nitrotyrosination. Thus, our data clearly revealed that iNOS protein is in close contact with ovulated oocyte, and these observations may be of

**Fig. 5.** NOS mRNA expression in ipsilateral and contralateral oviducts. The mRNA levels for each NOS were measured by real-time quantitative PCR. The 18S rRNA was used as an internal standard, and results are expressed as a ratio of NOS to 18S. The mRNA levels of nNOS (A), iNOS (B), and eNOS (C) were quantified in the isthmus, isthmic-ampullary junction, and ampulla of ipsilateral () and contralateral () oviducts at the postovulatory (d 0–3) and follicular (d 18–20) stages of the estrous cycle. Means with designations (*) are significantly different at <0.05 (Duncan’s test). Data are means ± SEM of eight animals.

**Fig. 6.** Effects of 17β-estradiol on NOS expression in the oviducts in vivo. The mRNA levels of NOS isoforms were measured by real-time quantitative PCR (LightCycler). The 18S rRNA was used as an internal standard and results are expressed as a ratio of NOS to 18S. Cows received either uterine infusion of saline control (CTL) or 17β-estradiol (E2) at d 14 of the estrous cycle. The mRNA levels of each gene were quantified in the whole oviducts from both groups. A, nNOS. B, iNOS. C, eNOS. Data are means ± SEM of three animals. Means with designations (*) are significantly different at <0.05 (Duncan’s test).
crucial importance because NO positively affects final oocyte maturation in vitro (30, 59). Furthermore, histological analysis has revealed that the epithelium of the bovine ampulla exhibits numerous ciliated cells (74). The activity of these cilia is greatest at or just after the time of ovulation and contributes to oocyte transport toward the site of fertilization (75). Interestingly, several studies demonstrated that NOS isoforms were expressed in different ciliated epithelium in mammals and that NO plays an important role in the control of ciliary activity in airway (21, 76). Indeed, it was notably shown that NO produced by iNOS up-regulates ciliary beat frequency in sinus epithelium (77). Therefore, our findings indicate that iNOS may also be involved in the increases of ciliary activity and the transport of the oocyte to the ampulla in the oviduct.

The heterogenous expression of NOS in the oviduct segment throughout the estrous cycle suggests that these enzymes are regulated by hormonal status at the transcriptional level. There are numerous reports that sex steroids, especially the estrogens, can regulate the mRNA expression of the NOS isoforms in a variety of cells (5, 7, 9). Consistent with estrogen regulation, our analysis clearly demonstrated that the expression of each NOS isoform increases during the maturation of the dominant follicle when maximal estrogen level is measured. Taken together, these findings prompted us to analyze the in vivo effects of the estradiol on the NOS mRNA expression in the bovine oviduct. Surprisingly, we found that estradiol selectively up-regulates iNOS expression, whereas nNOS and eNOS expression remains unchanged.

In accordance with these findings, our current observations demonstrated that iNOS expression is up-regulated in the isthmus region of the oviduct ipsilateral to the dominant follicle, which is known to contain higher estradiol levels. Recent studies also revealed that NOS isoforms are differently regulated by estradiol in a specific reproductive organ. Indeed, it was shown that the expression of nNOS and eNOS in the sheep uterus are differentially regulated by estrogen (21). Furthermore, estradiol increases iNOS expression and had no effect on eNOS expression in the rat uterus (78). Other reports indicated that iNOS expression was also up-regulated by estrogens in different cell types such as macrophages and cardiac myocytes (79, 80). It has been proposed that estrogen receptors-α and -β mediate transcriptional activation of iNOS by estradiol (81, 82). Interestingly, these receptors are known to be expressed in the bovine oviductal cells at all stages of the estrous cycle (83). We previously reported that 17β-estradiol also induces the transcription of GPx-4, an antioxidant gene, in the bovine oviductal epithelium (55). Because it is known that NO regulates GPx activity (84), this increase in iNOS expression should affect GPx-4 ability to neutralize products of lipid peroxidation in these cells. Furthermore, the estrogens are also known to regulate oviductal contraction and secretion in several species (85-87). Therefore, because iNOS produces a higher amount of NO than the other two low-output NOS isoforms, our data strongly suggest that the estradiol can significantly increases the NO concentration in the oviducts. We propose that this augmentation of NO may be of crucial importance for reproductive events that occur in the oviduct during the peri-ovulatory period.

In summary, we report that nNOS, iNOS, and eNOS are hormonally regulated and differentially expressed in the oviduct segments throughout the estrous cycle. We also provide the first information on NOS localization in the oviduct cell types. Thus, the dynamic expression and the specific cellular distribution of nNOS, iNOS, and eNOS along the oviduct during the estrous cycle allow us to suggest specific roles for each NOS isoform in oviductal functions. Furthermore, despite the fact that there are more evidences that NO is involved in all oviductal functions, very little is known about the mechanisms of control of NOS expression by extracellular signals in the mammalian oviduct. In this study, we show that estradiol selectively up-regulates iNOS expression and provide the first demonstration that NO production is regulated by a specific endocrine hormone in the oviducts in vivo. This finding suggests that NO produced by iNOS may modulate the numerous effects of estradiol in the oviduct. Collectively, our observations shed some light on the implication of each NOS isoforms in the physiological functions of the oviduct in vivo.

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