J. Vet. Sci. (2005), **6**(2), 97–101

JOURNAL OF
Veterinary
Science

Expression of nitric oxide synthase isoforms in the porcine ovary during follicular development

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The expression of nitric oxide synthase (NOS) isoforms in the ovaries of pigs was examined to study the involvement of nitric oxide, a product of NOS activity, in the function of the ovary. Western blot analysis detected three types of NOS in the ovary, including constitutive neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS); eNOS immunoreactivity was more intense compared with that of iNOS or nNOS. Immunohistochemical studies demonstrated the presence of nNOS and eNOS in the surface epithelium, stroma, oocytes, thecal cells, and endothelial cells of blood vessels. Positive immunoreactions for nNOS and iNOS were detected in the granulosa cells from multilaminar and antral follicles, but not in those of unilaminar follicles. iNOS was detected in the surface epithelium, oocytes, and theca of multilaminar and antral follicles. Taking all of the findings into consideration, the observed differential expression of the three NOS isoforms in the ovary suggests a role for nitric oxide in modulating reproduction in pigs.

Key words: granulosa cell, nitric oxide synthase, oocyte, ovary, pig

Introduction

Nitric oxide (NO) is a reactive free radical gas that is derived from L-arginine by the action of NO synthase (NOS) [15]. NO has diverse roles including intracellular signaling and vasoregulation [1], and exists in a variety of isoforms. A constitutive, calcium-dependent isoform (cNOS) is activated rapidly by agonists that elevate intracellular free calcium and is found in endothelial cells (eNOS) and the brain (nNOS) [7]. A calcium-independent inducible isoform (iNOS) can be induced after several hours of immunological stimulation and is detectable in macrophages, neutrophils, and endothelial cells [6].

Several studies have identified the presence of different isoforms of NOS in female reproductive tissues, including the ovary [16], oviduct [2], and uterus [8]. In addition, nitric oxide is known to be an important factors in the physiology and pathophysiology of reproduction [9].

In pigs, the expression of iNOS and eNOS has been studied in ovaries [11,12], in which iNOS was shown to be mainly localized in the oocytes, cumulus cells, and corpus luteum [12], whereas eNOS was detected by immunostaining in oocytes, granulosa cells, cumulus cells, corpus luteum, and corpus albicans [3,11,12]. Recently, many studies have suggested that nNOS, one of the constitutive isoforms of NOS, is found in non-neuronal cells, including macrophages [5]. This implies that nNOS, in addition to eNOS and iNOS, may contribute to the physiology of the ovary. However, little is known about the expression of nNOS in the ovary. The aim of this study, therefore, was to compare the expression patterns of eNOS, iNOS, and nNOS in the porcine ovary during follicular development in order to elucidate the phenotype of the cells in which each NOS isoform is expressed.

Materials and Methods

Tissue sampling

Ovary samples were collected from 6-month-old Landrace pigs at a local slaughterhouse, excluding pigs that were visually assessed as non-pregnant. Immediately after collection of each ovary, 0.5 cm pieces were dissected and placed at −70ºC until they were used for Western blotting analysis. Additional tissue pieces were processed for paraffin embedding after fixation in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4).

Histological analysis

Ovary tissues were sectioned (5 μm), deparaffinized in

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xylene, and rehydrated through a graded ethanol series to distilled water before staining with hematoxylin and eosin.

Follicle classification

Ovarian follicles were divided into three classes as described previously [13]: (1) unilaminar follicles (with one layer of granulosa cells), (2) multilaminar follicles (with multiple granulosa cell layers), and (3) antral follicles (with multiple granulosa cell layers enclosing an antrum). Nonatretic antral follicles had an intact membrana granulosa and no invagination of the theca layer into the granulosa layer. Atretic antral follicles had a thinner, fragmented granulosa cell layer.

Antibodies

The antibodies used in this study were as follows: mouse monoclonal anti-endothelial nitric oxide synthase (eNOS) antibody, rabbit anti-inducible nitric oxide synthase (iNOS) antisera, and rabbit anti-neuronal nitric oxide synthase (nNOS) antisera (all from BD Biosciences, USA).

Western blot analysis

Samples of porcine ovary were dissected free of extraneous tissue, homogenized in lysis buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and then centrifuged. Aliquots of the supernatants containing 40 μg of protein were separated on 8% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose transfer membranes (Schleicher & Schuell Bioscience, USA). The membranes were probed with anti-eNOS monoclonal antibody, rabbit anti-iNOS, or rabbit anti-nNOS antisera diluted 1 : 1000 in blocking solution. The reaction was visualized by labeling with horseradish peroxidase-conjugated horse anti-mouse IgG or anti-rabbit IgG secondary antibody (Vector, USA). The peroxidase reaction was developed with Amersham ECL reagents (Amersham Biosciences, USA). After imaging, the membranes were stripped and reprobed using monoclonal anti-beta-actin antibody as the primary antibody (Sigma, USA).

Immunohistochemistry

Immunostaining for eNOS, iNOS, and nNOS was performed as described previously [5]. Briefly, paraffin-embedded sections (5 μm) of porcine ovary were deparaffinized and treated with citrate buffer (0.01 M, pH 6.0) in a microwave for 2 min. The sections were treated with 0.3% hydrogen peroxide in methyl alcohol for 20 min to block endogenous peroxidase activity. After three washes in phosphate-buffered saline (PBS), the sections were incubated with 10% normal horse or goat serum and thereafter incubated with mouse anti-eNOS antibody, rabbit anti-iNOS, or rabbit anti-nNOS antisera (1 : 200 dilution) for 1 h at room temperature. After three washes in PBS, the appropriate biotinylated secondary antibody and avidin-biotin peroxidase complex (Vector Elite; Vector, USA) were added sequentially. The peroxidase reaction was developed with diaminobenzidine as a substrate (Vector, USA). Before being mounted, the sections were counterstained with hematoxylin. As a control, the primary antisera were omitted for a few test sections in each experiment, and no specific labeling of cell bodies or fibers was found in these sections (Fig. 3D).

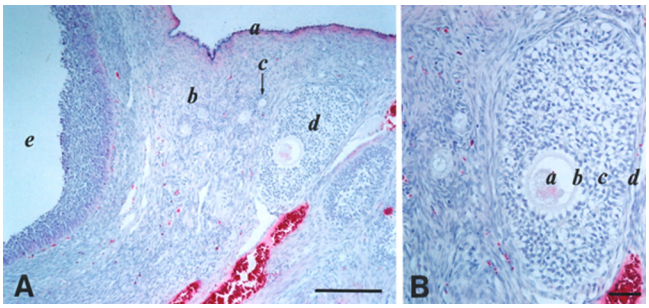

Fig. 1. Histological findings in porcine ovaries. A, Surface epithelium (a); stroma (b); unilaminar follicle (c); multilaminar follicle (d); antral follicle (e). Scale bar = 200 μm. B, Multilaminar follicle, oocyte (a); zona pellucida (b); granulosa cells (c); theca (d). Scale bar = 50 μm. H & E stain.

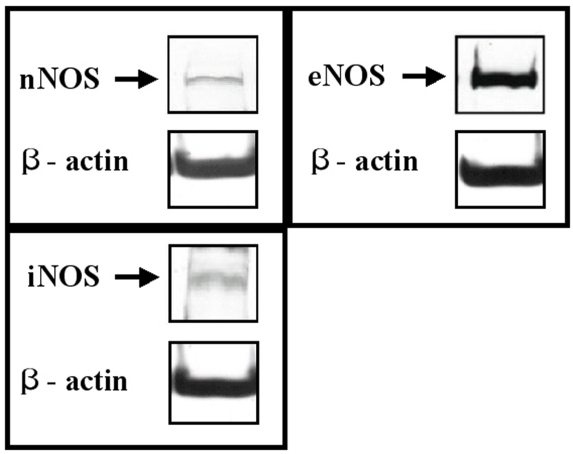

Fig. 2. Western blotting of nNOS, eNOS and iNOS in porcine ovaries. The ovaries proteins equivalent to 40 μg were separated on 8% SDS-PAGE, and analyzed by immunodetection using anti-eNOS antibody, nNOS or iNOS antisera. β-actin was used as a control. Arrows indicate the position of nNOS (155 kDa), eNOS (140 kDa), and iNOS (130 kDa), respectively.

Results

Histologic structure of the ovary

The ovarian tissue was divided into an outer cortex and an inner medulla. A simple squamous or cuboidal epithelium covered the cortex of the ovary. The cortical stroma contained

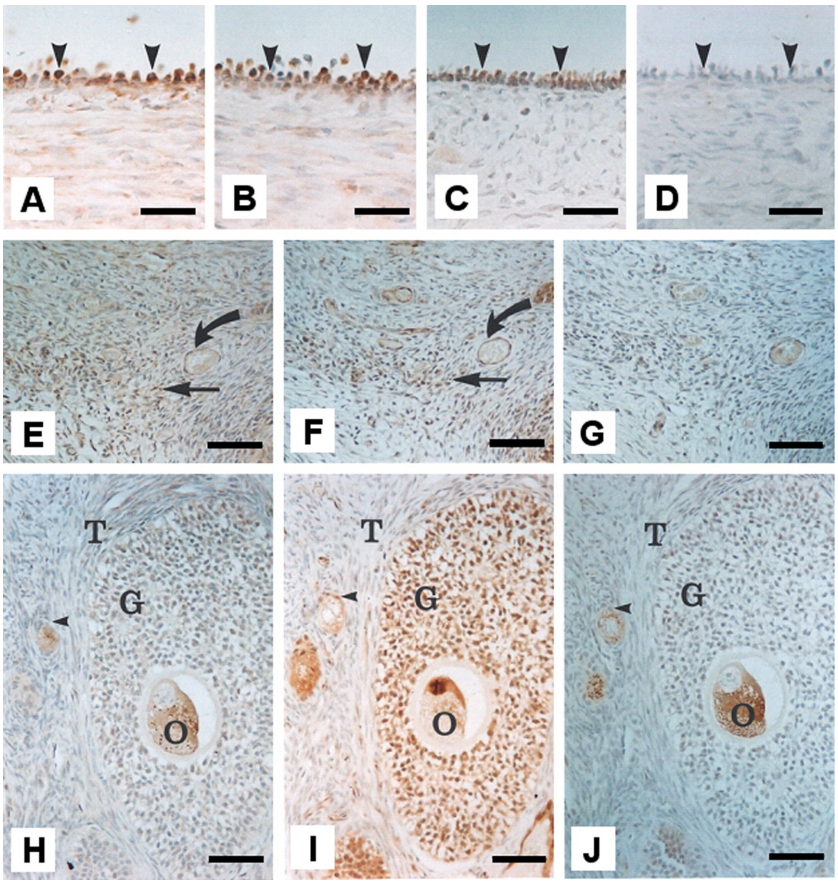

Fig. 3. Immunohistochemical localization of nNOS (A, E, H), eNOS (B, F, I), and iNOS (C, G, J) in porcine ovaries. G, granulosa; O, oocyte; T, theca. nNOS (A, E) and eNOS (B, F) were expressed in the surface epithelial cells (arrowheads), stroma cells (straight arrow), and vascular endothelial cells (curved arrow). iNOS (C) was also expressed in the surface epithelial cells (arrowheads). In unilaminar follicles, nNOS (H), eNOS (I), and iNOS (J) were expressed in the oocyte, and eNOS was expressed in the granulosa cells (I, arrowhead), while nNOS (H, arrowhead) and iNOS (J, arrowhead) showed no immunoreactivity in granulosa cells. nNOS (H) and eNOS (I) were expressed in the granulosa cells, oocytes, and theca interna of multilaminar follicles. iNOS (J) was expressed in the granulosa cells and oocytes of multilaminar follicles. No specific reaction product is seen in sections incubated with non-immune sera (D; arrowheads indicate the surface epithelial cells). A-J: Counterstained with hematoxylin. Scale bars: in A-D, 30 μm; in E-J, 60 μm.

the ovarian follicles. Unilaminar follicles, multilaminar follicles, and antral follicles were seen in the cortex (Fig. 1A). From the interior to exterior, the multilaminar follicle was comprised of the oocyte, zona pellucida, granulosa cells, and theca (Fig. 1B).

Western blot analysis of three isoforms of NOS in the ovary

The expression levels of nNOS, eNOS, and iNOS were assessed semiquantitatively by densitometry after Western blotting. Immunoreactivity for all three isoforms of NOS was detected in the porcine ovary; in particular, eNOS immunoreactivity was more intense relative to that of iNOS or nNOS (Fig. 2).

Immunohistochemical localization of nNOS, eNOS, and iNOS in the ovary

Expression of nNOS was detected in the surface epithelial cells (Fig. 3A), stromal cells (Fig. 3E), and the endothelial cells of blood vessels (Fig. 3E). In the unilaminar, multilaminar, and antral follicles, nNOS immunoreactivity was localized to the oocytes. Immunostaining for nNOS was present in the granulosa cells of multilaminar follicles, but was absent in those of unilaminar follicles (Fig. 3H). Moreover, a positive immunoreaction for nNOS was observed in the theca of multilaminar follicles (Fig. 3H). The expression of nNOS in the theca and granulosa cells of antral follicles (Fig. 4A) was strong compared with that in multilaminar follicles (Fig. 3H). In atretic follicles, nNOS immunoreactivity was localized to the fibrous theca layer.

The immunostaining pattern of eNOS was largely the same as that of nNOS; however, eNOS was additionally detected in the granulosa cells of unilaminar follicles (Fig. 3B, F, I; Fig. 4B).

Expression of iNOS was detected in surface epithelial cells (Fig. 3C). In the unilaminar, multilaminar, and antral

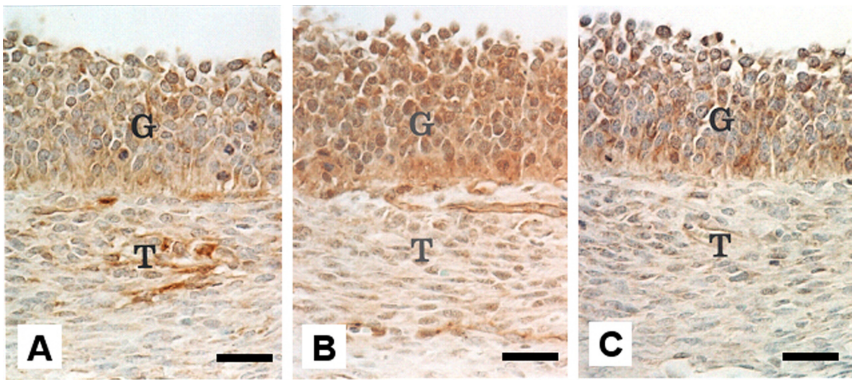

Fig. 4. Immunohistochemical localization of nNOS (A), eNOS (B), and iNOS (C) in the antral follicle. G, granulosa; T, theca. nNOS (A), eNOS (B), and iNOS (C) were expressed in the granulosa cells and theca of antral follicles. A-C: Counterstained with hematoxylin. Scale bars = 30 μm.

Table 1. Immunohistochemical localization of neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) isoforms of nitric oxide synthase (NOS) in the ovaries of pigs. The intensity of staining is indicated by (-), where staining was absent, up to (+++), for maximal staining

Tissue / cell type		nNOS	eNOS	iNOS
Surface epithelium		+	+	+
Intersitium	Stroma	+++	++	-
	Blood vessels			
	Endothelia	+	++	-
	Tunica media	-	-	-
Unilaminar follicles	Oocytes	+	+	+
	Granulosa cells	-	+	-
Multilaminar follicles	Oocytes	+	+	+
	Granulosa cells	++	+++	+
	Theca	+	+	-
Antral follicles	Oocytes	+	+	+
	Granulosa cells	+++	+++	++
	Theca	++	++	+
Atretic follicles	Fibrous theca	+	+	+

follicles, the iNOS immunoreactivity was localized to the oocytes. Immunostaining for iNOS was weakly detected in the granulosa cells of multilaminar follicles, but was not detected in those of unilaminar follicles (Fig. 3J). A positive immunoreaction for iNOS was present in the theca of antral follicles (Fig. 4C), but was absent in those of multilaminar follicles (Fig. 3J). In atretic follicles, iNOS immunoreactivity was localized to the fibrous theca layer (Table 1).

Discussion

This study was the first to demonstrate that three isoforms of NOS, including nNOS, eNOS, and iNOS, were expressed in porcine ovaries during follicular development. There is a general consensus that each NOS isoform is expressed in the ovarian follicles of pigs [11,12]. It has been shown that within large-sized follicles (7-10 mm in diameter) of porcine ovaries, eNOS is expressed in the oocytes, vascular endothelial cells, granulosa cells, theca cells, and cumulus cells; but no eNOS immunoreactivity is observed in the cumulus cells of medium follicles (3-6 mm in diameter) [12]. This suggests that eNOS expression is associated with stages of ovarian follicular development in pigs. In the present study, the observed patterns of eNOS immunostaining in the ovary were largely consistent with those of previous reports [11,12].

Although the expression of iNOS in porcine ovaries is well known, our findings contrast in part with the previous report [12]. In the present study, iNOS was mainly localized to the oocytes of unilaminar and multilaminar follicles, and to granulosa and theca cells. However, it was previously reported that iNOS, particularly in large follicles, was localized to the oocytes and cumulus cells [12]. This discrepancy might be a result of the different antisera used in the present study or a difference in the immunodetection methods used.

NOS has diverse functional roles in the ovary. The expression of NOS in the ovarian follicles implies that nitric oxide, generated from iNOS, is involved in the ovulatory process in rats [10]. This interpretation is further supported by the observation that inhibition of iNOS reduced ovulation by a maximum of 54% [10]. In addition, it is also suggested that eNOS [9,10] and nNOS, from the present findings, also participate in the process of ovulation.

The eNOS and iNOS isoforms (but not nNOS) have previously been immunolocalized to mammalian ovaries [4,12,14]. In this study, nNOS immunoreactivity was observed in the stroma, oocytes, theca cells, and granulosa cells of multilaminar and antral follicles. Recently, it has been accepted that nNOS is expressed in non-neuronal cells, including macrophages. However, the exact role that nNOS plays in the ovary remains to be determined.

The findings, together with previous research, indicate that the expression of NOS is in part dependent on the stage of ovarian follicle development. At the early stage of follicular development, little NOS immunostaining was detected in granulosa or theca cells. In the later stages, including Graafian follicles, immunostaining for three isoforms of NOS was detected in the granulosa and theca cells. This finding suggests that, in the porcine ovary, granulosa and theca cells may serve as sources of nitric oxide.

In conclusion, this study revealed that three isoforms of NOS were expressed in the porcine ovary, suggesting that nitric oxide might be involved in the process of follicular development and/or the ovulatory process.

Acknowledgments

This research was supported by the Program for the Training of Graduate Students in Regional Innovation which was conducted by the Ministry of Commerce Industry and Energy of the Korean Government.

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