—Original—

Expression of Nitric Oxide Synthase-3 in Porcine Oocytes Obtained at Different Follicular Development

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Abstract. The present study was designed to determine the localization of nitric oxide synthase-3 (NOS-3) in porcine follicles during follicular development. A 130-kDa NOS-3 protein was found with greater frequency much in the oocytes than in the cumulus cells, as revealed by Western blotting analysis. The content of NOS-3 in the oocyte was higher in large follicles (> 7-mm diameter) than in small follicles (< 2-mm). The data by Western blotting showed the same pattern as the observations obtained from the immunohistochemical studies, in which the periphery of the oocyte stained strong positive. The inner surface cell layer of granulosa cells and cumulus cells were positive staining, especially in large antral follicles. In the primordial follicles, NOS-3 was restricted to the cytoplasm of oocytes, and no stained product was observed in the nucleus of oocytes or granulosa cells. A significant synthesis of NO by oocytes was observed in the presence of ionomycin, but not in the absence of ionomycin, indicating that oocyte NOS-3 functions in response to transient elevations in the intracellular calcium level. We concluded that NOS-3 is expressed in the oocyte from the primordial follicular stage to antral follicular stage, and that it is functional at least in the antral follicles.

Key words: Nitric oxide, Oocyte, NOS-3, Immunohistochemistry, Follicular development
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Nitric oxide (NO) is generated by three isoforms of NO synthase, neural (NOS-1), inducible (NOS-2) and endothelial (NOS-3) synthases, and mediates a variety of cellular functions. The expression of mRNAs and proteins for NOS-2 and NOS-3 has been investigated in the rat ovary during follicular development, ovulation and pseudopregnancy [1, 2]. NO generation has also been found in immature ovaries [3, 4] and at various stages of follicular development [5, 6]. Using porcine granulosa cell culture, it was shown that FSH could promote expression of the NOS-3 gene and NO synthesis during cell maturation [7,

8]. These reports indicate that NO synthesis in ovarian cells is principally dependent upon gonadotropin stimulation. Recent studies on the function of NO in oocytes have attracted interest. The presence of NOS-3 in the oocyte has been reported in rat [2] and porcine [9, 10]. An immunohistochemical study of immature and gonadotropin-stimulated follicles demonstrated the presence of NOS-3 in the peripheral region of rat oocytes. Western blotting and reverse transcription-polymerase chain reaction of porcine oocytes revealed the presence of NOS-3 protein and its mRNA. The lack of NOS-3 impairs oocyte meiotic maturation and ovulation [11, 12]. A large

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amount of NO produced by oocyte NOS-3 may be released as a paracrine factor [10]. However, there were little literatures concerning the localization of NOS-3 in ovarian follicles during follicular development. It is still not known whether oocyte NOS-3 has the ability to synthesize NO. The purpose of the present study was to determine the localization of NOS-3 in porcine follicles at the stages of the primordial follicle, the early antral follicle and the mature follicle, and to estimate NO synthesis by the oocytes.

Materials and Methods

Culture of cumulus-oocyte complexes

Ovaries were obtained from gilts at a local slaughterhouse. Cumulus-oocyte complexes (COCs) were obtained from different size follicles (< 2-mm, 3–6 mm, >7-mm diameter) by aspiration with an 18-gauge needle, and washed four times with Hanks balanced salt solution supplemented with 1 mg/ml bovine serum albumin (BSA). In some experiments, COCs from medium size follicles (3–6 mm) were washed with culture medium, phenol-red free DMEM/Ham's F12 (GIBCO-BRL Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10 nM estradiol-17 β (E₂), 10 μ g/ml insulin, 5.5 μ g/ml transferrin, 6.7 μ g/ml selenite, 1 mg/ml BSA, 10% steroid-free fetal bovine serum (GIBCO-BRL Life Technologies) and 50 μ g/ml gentamicin. Thirty to forty COCs were seeded into a 100- μ l drop of culture medium in a 4-well dish (Nunc Multidishes; Nalge Nunc International, Denmark) under mineral oil. COCs were cultured for 15 h in a humidified atmosphere of 95% air and 5% CO₂.

Western blotting analysis

Oocytes and cumulus cells were separated by gentle pipetting, then they were suspended in 10 mM Tris buffer (pH 6.8) containing 5 mM EDTA. Cumulus cells were frozen-thawed twice, and the cell suspension was centrifuged at 20,000 $\times$ g for 20 min, then the soluble fraction was used for electrophoresis. Oocyte and cumulus cells proteins were analyzed by Western blotting using anti-NOS-3 antiserum (Alexis Biochemicals, San Diego, CA, U.S.A.) as previously described [9].

NO synthesis by oocytes

Oocytes were denuded of cumulus cells by gentle pipetting and washed twice with PBS. Ten oocytes were added to a 1.5-ml plastic tube containing 1 μ M ionomycin (Sigma) in 100- μ l culture medium (without fetal bovine serum). The plastic tube was covered with a lid, and then a small hole was made in the wall of each tube with an 18-G needle, because of the oxygen requirement of NO synthesis. Each group, composed of test and blank (no oocytes) tubes, was carried out in triplicate. After a 3-h incubation under 95% O₂ and 5% CO₂ at 38.5 C, each hole was covered with plastic tape, and the tubes were centrifuged at 15,000 g for 15 sec.

Measurement of nitrite and nitrate

Concentrations of NO_x (nitrite and nitrate) were measured with an automated NO detector high-pressure liquid chromatography system (ENO-20; EICOM, Kyoto, Japan) as described in our previous report [9] with minor modifications. In brief, after the removal of proteins with 50% ice-cold methanol, 20 μ l soluble fraction were loaded into the automated NO detector. A reverse-phase separation column packed with polystyrene polymer (NO-PASK, 4.5 $\times$ 50 mm) separated nitrite and nitrate, and nitrate was reduced to nitrite with copper-plated cadmium filings. Nitrite was mixed with Griess reagent to form a purple azo dye in a reaction coil. The mobile phase was 10% methanol containing 0.15 M NaCl/NH₄Cl and 0.5 mg/ml EDTA-4Na, and flowed at a rate of 0.33 ml/min. The absorbency of the product dye was measured at 540 nm by a flow-through spectrophotometer. The contamination of NO_x was estimated in the ionomycin-containing medium without oocytes.

Immunohistochemistry

The ovaries were fixed with 4% paraformaldehyde in PBS. The materials were embedded in paraplast (Oxford Lab., MD, USA), cut into sections 5 μ m thick and attached on glass slides that were treated with Biotin Meshcement (Oken, Tokyo, Japan). The specimens were treated with xylene and a descending series of alcohol for the deparaffinization, then were used for immunohistochemistry. We utilized the conventional avidin-biotin complex (ABC) method for the immunohistochemical staining which has been described elsewhere [13, 14]. Briefly, the specimens were treated with 0.3% H₂O₂ in

methanol, with 0.5 M glycine in PBS and then with 0.2% triton-X100 in PBS. After blocking the non-specific reaction with 1% BSA and 1% normal goat serum in PBS, the specimens were incubated with anti-NOS-3 antiserum at a dilution of 1: 60 with the blocking solution for 3 h at room temperature. Only PBS was substituted for primary antiserum for immunological control, and no specific immunoreactivity was detected in the control specimens. After washing with PBS, the specimens were treated with standard secondary antibody for the ABC method (Vector Lab., CA, USA). The immunoreactivity was visualized by exposing the specimen to PBS buffered 0.05% diaminobenzidine (DAB) activated with 0.002% H₂O₂ for several minutes. The specimens were mounted in Mount Quick (Daido Sangyo Co., Tokyo, Japan) and observed with a Nikon E-800 light microscope.

Statistical analysis

The data are expressed as means $\pm$ S.D., and differences between them were evaluated using Student's *t* test following one-way ANOVA. $P < 0.05$ was considered significant.

Results

Oocyte and cumulus cell proteins prepared from small (< 2-mm in diameter) and large (> 7-mm) antral follicles of porcine ovaries were analyzed by Western blotting using anti-NOS-3 antiserum. As shown in Fig. 1, a single band of NOS-3 positive protein with an apparent mol. wt. of 130 kDa was detected in two oocyte samples. When cellular proteins equivalent to 30 COCs were subjected to Western blotting, however, the cumulus cells showed little immunostaining for the 130-kDa protein band. The intensity of oocyte NOS-3 immunostaining was higher in large follicles than in small follicles. These findings suggest that the level of oocyte NOS-3 increases during follicular development.

We tested whether oocytes had the ability to synthesize NO. COCs prepared from medium-sized follicles (3–6 mm) were cultured for 15 h, and then their denuded oocytes were treated with ionomycin, which induces transient elevations in intracellular calcium level. Oocytes showed significant synthesis of NO in response to ionomycin, whereas little NO synthesis was

Fig. 1. Western blotting of NOS-3 isoform from porcine cumulus cells and oocytes. Cumulus cells (lanes 1 and 2) and oocytes (lanes 3 and 4) were prepared from small (< 2-mm in diameter) and large (> 7-mm) antral follicles of porcine ovaries as described in Materials and Methods. The cellular proteins equivalent to 30 COCs were separated on SDS-PAGE, and analyzed by immunodetection using anti-NOS-3 antiserum. Data are representative of three independent experiments performed for two different protein samples. (B) The band intensity of NOS-3 was quantified using a densitometry program (NIH Image). Data are expressed as means $\pm$ S.D. ($n=3$).

observed in the absence of ionomycin (Fig. 2). These results indicate that NOS-3 detected in porcine oocytes has the ability to synthesize NO, at least after 15 h culture of COCs.

Since oocyte NOS-3 in healthy follicles was functional, the localization of NOS-3 was immunohistochemically investigated using the

Fig. 2. Oocyte NO synthesis after treatment of COCs with E₂. COCs prepared from medium-sized follicles (3–6 mm) of porcine ovaries were cultured for 15 h with 10 nM E₂. Oocytes were recovered after removal of cumulus cells, and then 10 oocytes were incubated for 3 h in 100 μ l culture medium with or without 1 μ M ionomycin as described in Materials and Methods. The net NOx level was determined by subtracting the blank (no oocytes). Data are expressed as means $\pm$ S.D. (n=3).

primordial, small (early antral stage) and large (later antral stage) follicles. In early antral follicles, oocytes showed NOS-3-positive (Fig. 3A). In particular, the peripheral regions of oocytes showed strong positive staining. The zona pellucida and lipid droplets in oocytes stained negative. On the other hand, the inner surface cell layer of granulosa cells and cumulus cells stained positive. In addition, the vascular endothelial cells also showed positive staining. In later antral follicles, increased staining was observed in oocytes, the inner surface cell layer of granulosa cells and cumulus cells (Fig. 3B). The increased staining in oocytes followed the pattern of the results shown by Western blotting in Fig. 1. In the primordial follicles, immunoreactivity for NOS-3 was restricted to the cytoplasm of oocytes, and no staining product was observed in the nucleus of oocytes and granulosa cells (Fig. 3C). In control sections, no staining product was observed when

Fig. 3. Immunohistochemical localization of NOS-3 in porcine ovaries. Porcine ovaries were collected as described in Materials and Methods, and 5- μ m sections of the ovaries were stained by the conventional avidin-biotin complex method. (A) The small antral follicle at the early antral stage showed NOS-3-positive oocytes. Cumulus cells and the inner surface of the granulosa cell layer (arrow) were also stained. The vascular endothelial cells (asterisk) also showed positive staining. (B) In the large antral follicle, the intensities of NOS-3 immunostaining in oocyte, cumulus cells and the inner surface of the granulosa cell layer (arrow) were increased. (C) In the primordial follicles, the immunoreactivity for NOS-3 was restricted to the cytoplasm of oocytes (arrows). O, oocyte; C, cumulus cell; G, granulosa cell layer; T, theca cell layer. A and B, scale bar = 100 μ m. C, scale bar = 20 μ m.

PBS was substituted for the primary antibody for immunological control.

Discussion

Immunoreactive NOS-3 was detected on the surface of rat oocytes as well as in gonadotropin-stimulated theca and granulosa cells [2]. In our previous study [9], a 130-kDa NOS-3 protein was also shown in porcine oocytes as revealed by Western blotting analysis. The present study showed that immunoreactive NOS-3 was present in the primordial follicle and that expression of oocyte NOS-3 increased during follicular development. There were differences in the content of 130-kDa NOS-3 protein between small (< 2-mm in diameter) and large (> 7-mm) antral follicles. Immunohistochemical studies also showed that the intensity of NOS-3 immunostaining was higher in the oocytes of large antral follicles than in those of small antral follicles. Consequently, the present results support the point of view that expression of oocyte NOS-3 is influenced by gonadotropin at the antral follicular stage. In early antral follicles, granulosa and cumulus cells showed positive immunostaining, but the granulosa cells were negative in the primordial follicles. As previously reported using culture of porcine granulosa cells [8], expression of granulosa cell NOS-3 is constant after 12 h of FSH stimulation. Gonadotropin treatment is known to induce NOS-3 expression in the theca and granulosa cells [1, 2]. The immunoreactivity for NOS-3 shown in the granulosa and cumulus cells of early antral follicles may be the result of gonadotropin stimulation. It is worthy of note that the oocytes of the primordial follicles showed positive staining, since gonadotropin stimulation does not occur in the primordial follicle. At the antral follicular stage, an increase in the level of oocyte NOS-3 may be the result of gonadotropin stimulation. Although NOS-3 is present in the oocyte of the primordial follicle, its expression may not be directly regulated by gonadotropin. The role of estrogen in the expression of NOS-3 has been reported. The amount of NOS-3 protein in aortic tissues markedly decreases in ovariectomized rats, but it can be reversed by oral administration of E₂ valerate for 14

days [15]. Chronic estrogen treatment also increases the levels of eNOS protein in rat cerebral microvessels [16]. In addition, recent reports support the concept that NOS expression is not necessarily related to the acute release of NO. For example, the acute response of NOS-3 to E₂ is replicated in COS-7 cells cotransfected with the estrogen receptor and NOS-3, but not in those transfected with NOS-3 alone [17]. It is unclear whether the primordial follicles are regulated by estrogen.

It was shown that oocyte NOS-3 has the ability to synthesize NO, at least in the antral follicles. Oocytes prepared from medium-sized follicles produced NO in the presence of ionomycin, which induces transient elevations in the intracellular calcium level. However, the amount of NO synthesized was very low. This finding indicates a characteristic of oocyte NOS-3 which requires calcium for activity. It is considered that NOS-3 produces a small amount of NO in response to direct stimulus [18, 19]. Since the oocyte contains a high level of NOS-3, compared to other somatic cells, the oocyte may have the ability to produce a high amount of NO. NO negatively regulates steroidogenesis in the gonads [20–22, 5]. A large amount of NO produced by oocyte NOS-3 may be released as a paracrine factor [10]. In addition, a large amount of NO may be required for oocyte meiotic maturation and ovulation, because they are impaired in NOS-3-deficient female mice [11, 12]. However, the factor(s) inducing transient elevations in intracellular calcium level remains to be elucidated.

In conclusion, the present study indicates that NOS-3 is expressed in the oocyte from the primordial follicular stage to antral follicular stage and that oocyte NOS-3 is functional, at least in the antral follicles.

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