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Nitric oxide synthase isoforms and the effect of their inhibition on meiotic maturation of porcine oocytes

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Summary

In this paper we assessed: (i) the change in nitric oxide synthase (NOS) isoforms' expression and intracellular localization and in NOS mRNA in porcine oocytes during meiotic maturation; (ii) the effect of NOS inhibition by N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AG) on meiotic maturation of cumulus–oocyte complexes (COC) as well as denuded oocytes (DO); and (iii) nitric oxide (NO) formation in COC. All three NOS isoforms (eNOS, iNOS and nNOS) and NOS mRNA (eNOS mRNA, iNOS mRNA and nNOS mRNA) were found in both porcine oocytes and their cumulus cells except for nNOS mRNA, which was not detected in the cumulus cells. NOS isoforms differed in their intracellular localization in the oocyte: while iNOS protein was dispersed in the oocyte cytoplasm, nNOS was localized in the oocyte cytoplasm and in germinal vesicles (GV) and eNOS was present in dots in the cytoplasm, GV and was associated with meiotic spindles. L-NAME inhibitor significantly suppressed metaphase (M)I to MII transition (5.0 mM experimental group: 34.9% MI, control group: 9.5% MI) and at the highest concentration (10.0 mM) also affected GV breakdown (GVBD); in contrast also AG inhibited primarily GVBD. The majority of the oocytes (10.0 mM experimental group: 60.8%, control group: 1.2%) was not able to resume meiosis. AG significantly inhibited GVBD in DO, but L-NAME had no significant effect on the GVBD of these cells. During meiotic maturation, NO is formed in COC and the NO formed by cumulus cells is necessary for the process of GVBD.

Keywords: Cumulus cells, Meiotic maturation, NOS, Oocyte, Pig

Introduction

Mammalian oocytes must undergo meiotic maturation before they can be successfully fertilized. Meiotic maturation begins in the prenatal period and is soon interrupted at the late diplotene stage. After the period of growth, oocytes at the germinal vesicle stage (GV) can resume their meiotic maturation and pass through germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII), when meiotic maturation is again interrupted. The resumption of meiosis is induced, in *in vivo* conditions, by luteinizing hormone, or, under *in vitro* conditions, only by removal of the oocyte from the follicle (Wassarman, 1988). It is well known that meiotic maturation is regulated by factors such as maturation-promoting factor (MPF) or mitogen-activated protein kinase (MAPK) (Palmer *et al.*, 1998; Meinecke and Krischek, 2003; and others). However, other factors are also involved in its regulation. One of these is nitric oxide synthase (NOS), the enzyme that catalyses the synthesis of the important cell messenger, nitric oxide (NO), from L-arginine and molecular oxygen (Kwon *et al.*, 1990; Lamas *et al.*, 1992).

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NOS exists as three isoforms. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are calcium and calmodulin dependent and both synthesize a small amount of NO. They were first identified in the endothelium (Lamas *et al.*, 1992) and the brain (Bredt *et al.*, 1991). The third isoform, inducible NOS (iNOS), is calcium independent and produces a 100 to 1000 times larger amounts of NO and for a longer time. It is expressed in many cells, e.g. macrophages, hepatocytes, etc., as a response to endotoxins and cytokines (Moncada *et al.*, 1991; Nathan, 1992).

Two of the known NOS isoforms, eNOS and iNOS, were reported to be present in mammalian ovaries. They were found in granulosa cells (Graselli *et al.*, 2001; Mitchel *et al.*, 2004; Tao *et al.*, 2004; and others), in cumulus cells (Van Voorhis *et al.*, 1995; Jablonka-Shariff & Olson, 2000; Mitchel *et al.*, 2004) and in oocytes (Takesue *et al.*, 2003). The presence of eNOS and iNOS was observed in mouse and rat oocytes (Van Voorhis *et al.*, 1995; Jablonka-Sharif & Olson, 1997, 2000; Mitchel *et al.*, 2004). Hattori *et al.* (2000) found the eNOS isoform in mature pig oocytes and Takesue *et al.* (2003) described the presence of this isoform in cumulus cells as well. However, the presence of nNOS protein has not been reported in oocytes. A high level of nNOS mRNA was found only in mouse oocytes (Abe *et al.*, 1999). The presence of mRNA for all three isoforms was recorded in cattle oocytes (Tesfaye *et al.*, 2006).

NOS isoforms are involved in the production of NO in various aspects of reproduction functions. They play a role in the regulation of gonadotropin secretion, spermatogenesis (Zini *et al.*, 1996; Gregg, 2003) and in fertilization (Kuo *et al.*, 2000). In the ovary, NOS isoforms are involved in the regulation of steroidogenesis (Van Voorhis *et al.*, 1995; Jablonka-Sharif & Olson, 1998) and folliculogenesis (Jablonka-Sharif & Olson, 1997). NOS activity and NO presence are necessary for the correct course of meiotic maturation in the oocyte (Jablonka-Sharif & Olson, 1998, 2000; Sengoku, 2001; Bu *et al.*, 2003; Viana *et al.* 2007).

The e-NOS knockout impairs the course of meiotic maturation of mouse oocytes *in vivo* (Jablonka-Shariff & Olson, 1998). The oocytes are unable to complete meiotic maturation to the metaphase II (MII) stage and they stop at metaphase I or they develop abnormally. The presence of NOS inhibitors in culture medium produces a similar effect on mouse oocytes *in vitro* (Bu *et al.*, 2003). The same effect of NOS inhibition has also been described in the oocytes of other mammal species – rats (Sela-Abramovich *et al.*, 2008) and cattle (Bilodeau-Goeseels, 2007; Schwarz *et al.*, 2008). NO is important for meiotic maturation of porcine oocytes too (Hattori *et al.*, 2000; Tao *et al.*, 2004, 2005).

NO is a highly diffusible gas (Moncada *et al.*, 1991) and thus NO originating from cumulus cells can influence oocyte meiotic maturation. However, no detailed information is available about the effect of NO that has originated in cumulus cells on meiotic maturation of porcine oocytes, as well as about the expression of NOS isoforms and NOS mRNA in the oocytes and cumulus cells during meiotic maturation.

The objective of this study was to investigate: (i) the changes of NOS isoforms' expression and NOS mRNA in porcine oocytes and cumulus cells and intracellular changes in the localization of NOS isoforms in relation to the stage of meiotic maturation; (ii) the influence of NOS inhibition by N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AG) on meiotic maturation and NO formation; and (3) the role of NO originating from cumulus cells in the meiotic maturation of porcine oocytes.

Materials and methods

Isolation and culture of oocytes

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the estrous cycle and transported to the laboratory within 1 h in a saline solution (0.9% sodium chloride) at 39 °C. Fully grown oocytes were collected from follicles by aspirating follicles that measured 2–5 mm in diameter with a 20-gauge needle. Only oocytes with compact cumuli were selected for further study. Before culture, the oocytes were washed three times in a maturation culture medium. The oocytes were cultured in a modified M199 medium (Gibco BRL, Life Technologies) containing sodium bicarbonate (32.5 mM), calcium L-lactate (2.75 mM), gentamicin (0.025 mg/ml), HEPES (6.3 mM), 13.5 IU equine chorionic gonadotropin (eCG), 6.6 IU human chorionic gonadotropin (hCG)/ml (P.G.600 Intervet) and 10% (v/v) fetal calf serum (Gibco BRL, Life Technologies). The oocytes were cultured for 0, 24 and 48 h in 3.5-cm diameter Petri dishes (Nunc) containing 3.0 ml of the culture medium at 39 °C in a mixture of 5.0% CO_2 in air.

Evaluation of oocytes

At the end of the culture, the cumulus cells were removed by repeated pipetting through a narrow glass pipette. Then the oocytes were mounted on slides, fixed with acetic alcohol (1:3, v/v) for at least 24 h and stained with 1.0% orcein. The oocytes were examined under a phase contrast microscope and the stages of meiotic maturation were determined.

Culture of oocytes for the determination of NO

For the determination of NO, the oocytes were cultured (0, 24 and 48 h) in NCSU 23 medium containing NaCl (1.088 mM), KCl (0.048 mM), $CaCl_2.2H_2O$ (0.017 mM), KH_2PO_4 (0.012 mM), $MgSO_4.7H_2O$ (0.012 mM),

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$NaHCO_3$ (0.239 mM), glucose (0.056 mM), glutamine (0.01 mM), taurine (0.054 mM), hypotaurine (0.05 mM) and bovine serum albumin (BSA; 0.4% w/v). The cumulus–oocyte complexes (COC) were cultured in Petri dishes (Nunc) containing 300 µl of the culture medium at 39 °C in a mixture of 5.0% CO_2 in air. Cumulus–oocyte complexes were homogenized together with NCSU 23 medium and proteins were removed by boiling and centrifugation.

Western blot

After culture, the oocytes were denuded of their cumulus cells by pipetting through a fine-bore pipette and lysed in an sodium dodecyl sulfate (SDS) sample buffer. Oocyte proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE with a 4% stacking gel and a 10% separating gel and then electrophoretically transferred onto a nitrocellulose membrane (Hybond). After blocking for 1 h with low-fat milk (5%) in Tris-buffered saline containing 0.1% Tween-20, the membrane was incubated overnight with primary rabbit anti-NOS antibodies (anti-eNOS, anti-iNOS, anti-nNOS, dilution 1:200 000, Alexis Biochemicals). The membrane was then incubated with peroxidase-conjugated anti-rabbit IgG (dilution 1:120 000, Calbiochem). The proteins were detected using the ECL western blotting analysis system (Amersham).

Laser-scanning confocal microscopy

The oocytes were denuded by pipetting with a glass pipette and their zonae pellucidae were removed by the treatment in 0.1% (w/v) pronase (Sigma–Aldrich). Then the oocytes were washed three times in phosphate-buffered saline (PBS)–BSA and fixed in 2.5% (w/v) paraformaldehyde in PBS for 60 min. After treatment in PBS–BSA–Triton X-100 (Sigma–Aldrich) for 2 h, they were incubated in PBS–BSA Tween 20 (Sigma–Aldrich) containing rabbit monoclonal anti-eNOS, anti-iNOS and anti-nNOS (1:100, Alexis Biochemicals) at 4°C overnight. After being washed three times in PBS–Tween 20 (10 min each), the oocytes were incubated in PBS–BSA–Tween 20 containing fluorescein 145 isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:100, Calbiochem) for 60 min at room temperature. Negative control experiments were performed by first antibody omission. After repeated washing in PBS–Tween 20 (three times, 10 min each), the DNA was stained with Hoechst 33258 (0.5% w/v, Sigma–Aldrich, 10 min). The oocytes were mounted on slides using the SlowFade antifade kit (Invitrogen) and observed under a laser scanning confocal microscope (Leica TCS SPE, Leica Microsystems).

Reverse transcription (RT)-PCR analysis for NOS mRNA

The oocytes were cultured for 0, 24 and 48 h. RNA was isolated separately from 50 oocytes and their cumulus cells. Total RNA was isolated using Total RNA – chemistry (ABI) in an Applied Biosystems 6100 PrepStation, in accordance with manufacturer's instructions. Total RNA was transcribed to cDNA with a High-Capacity cDNA Archive kit (ABI), in accordance with the producer's manual and frozen at –20 °C. cDNA was synthesized in a final volume of 10 µl. Sets of specific primers were synthesized in accordance with known sequences to amplify specific products for nNOS, iNOS and eNOS. nNOS sense (forward) primer 5′-TCAAGGTCAAGAACTGGGAG-3′, antisense (reverse) primer 5′-CCTGCAGCTTGGACCACTGG-3′ (Solhaug *et al.*, 2001), iNOS forward 5´-GCC GACTGGATTTGGTTGGT-3′, reverse 5′-GTTGGTGA GTTCTTTCAGCAT-3′ (Adrian *et al.*, 1999), eNOS forward 5′-AGCGGCTGCATGACATTGAG-3´, reverse 5′-AAAAGCTCTGGGTGCGTATGCG-3′ (Takesue *et al.*, 2001). Classical PCR was first conducted to confirm the specificity of the primers. The PCR products were separated using agarose gel electrophoresis and then visualized using ethidium bromide staining. The reactions were performed in a 10 µl reaction mixture containing 0.1–5 ng RNA (cDNA), 5 pmol of primers and 1× concentrated PPP Master Mix (150 mM Tris–HCl, pH 8.8, 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5mM $MgCl_2$, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 400 µM dTTP, 100/ml *Taq*–Purple DNA polymerase, stabilizers and additives – Top Bio, Czech Republic). The RT-PCR were performed as follows: initial denaturation (95 °C, 3 min and 0.5 min) followed by 35 cycles (95 °C, 30 s; 55 °C, 45 s and 72 °C, 30 s) for nNOS, 35 cycles (95 °C, 30 s; 60 °C, 45 s and 72 °C, 30 s) for iNOS and 40 cycles (95 °C, 30 s; 54 °C, 45 s and 72 °C, 30 s) for eNOS. The final extension step was at 72 °C for 3 min.

Determination of NO

Nitric oxide was determined by colorimetric measurement of NO metabolites, nitrites and nitrates (NO_3^-/NO_2^-). The colorimetric nitric oxide assay kit, (Calbiochem), was used for their determination. Nitrates were enzymatically converted by NO reductase into nitrites. Nitrites were quantified using Griess reagents (*p*-aminobenzenesulfonamide in 3.0 N HCl and *N*-(1-naftyl)ethylendiaminedichloride). The amount of nitrites was measured spectrophotometrically on microtitration plates using a Rainbow ELISA plate reader (wavelength 540 nm, SLT). The calibration curve was based on eight standard concentrations of nitrites (1.0 pmol/µl KNO_3 is equivalent to 500.0 µM NO).

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Experiment design

Experiment 1. The detection of NOS isoforms and NOS mRNA in the oocytes and their cumulus cells and intracellular localization of NOS isoforms in the oocytes during meiotic maturation

The oocytes were cultured for 0, 24 and 48 h to three stages of meiotic maturation (GV, MI and MII). eNOS, iNOS and nNOS and eNOS mRNA, iNOS mRNA and nNOS mRNA were determined in the oocytes and their cumuli.

Experiment 2. The effect of L-*NAME on the meiotic maturation of pig oocytes*

The oocytes were cultured for 48 h in the presence of the nonspecific NOS inhibitor L-NAME (Sigma–Aldrich) in concentrations of 1.0, 2.5, 5.0 and 10.0 mM. The control oocytes were cultured in the presence of an inactive form of NOS inhibitor, D-NAME (1.0, 2.5, 5.0 and 10.0 mM; Sigma–Aldrich) and in an inhibitor-free medium. The most effective concentration (10.0 mM) was used in further experiments.

Experiment 3. The effect of AG on the meiotic maturation of porcine oocytes

The oocytes were cultured for 48 h in the presence of the iNOS specific inhibitor AG (Sigma–Aldrich) in concentrations of 2.5, 5.0, 7.5 and 10.0 mM. The control oocytes were cultured in an inhibitor-free medium. The most effective concentration (10.0 mM) was used in further experiments.

Experiment 4. The effect of NOS inhibition on GVBD and MI–MII transition and the role of cumulus cells in GVBD of oocytes influenced by NOS inhibitors

The oocytes were cultured in medium with 10.0 mM AG or 10.0 mM L-NAME. The control oocytes were cultured in an inhibitor-free medium or a medium with an inactive form of the inhibitor, D-NAME (10.0 mM). For the determination of the effect of NOS inhibition on GVBD, the oocytes were cultured with the inhibitors for 24 h. To determine of the effect of NOS inhibition on MI–MII transition, the oocytes were cultured in an inhibitor-free medium for the first 24 h and then in a medium with inhibitors for the following 24 h. For detection of the role of cumulus cells in GVBD of oocytes subjected to a NOS inhibitor, the oocytes were denuded of their cumulus cells (DO) and cultured in the presence of an inhibitor for 24 h.

Experiment 5. NO formation in cumulus–oocyte cell complexes (COC) during meiotic maturation and the effect of AG on NO formation

Cumulus–oocyte cell complexes (60 COC in each group) were cultured for 0, 24 and 48 h in an inhibitor-free medium or in a medium with AG (5.0 and 10.0 mM). NO was measured using the colorimetric nitric oxide assay kit (Calbiochem) as described above. To eliminate the effect of AG and medium components, inhibitor-free medium and medium containing AG (5.0 and 10.0 mM) without COC were cultured. Nitrate and nitrite concentrations did not change in these media during cultivation (data not shown).

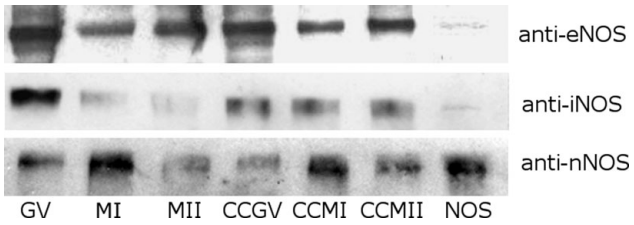

Figure 1 Immunoblotting of NOS isoform in porcine oocytes and their cumulus cells. Oocytes were cultured for 0, 24 and 48 h. Proteins were separated on SDS-PAGE, transferred to a nitrocellulose membrane and then incubated with NOS-specific antibodies (anti-eNOS, anti-iNOS, anti-nNOS). One sample contained proteins from 50 oocytes or their cumulus cells. GV, MI, MII – oocytes culture for 0, 24 and 48 h, CCGV, CCMI, CCMII – cumulus cells of oocytes cultured 0, 24 and 48 h.

Statistical analysis

Data from all the experiments were subjected to statistical analysis. All the experiments were repeated four times. The differences between the experimental and control groups of oocytes were evaluated by analysis of variance (Scheffe's test) using the statistical STATISTICA software package (STATISTICA 8.0, StatSoft). A p-value of less than 0.05 was considered to be statistically significant.

Results

Experiment 1. The detection of NOS isoforms and NOS mRNA in oocytes and their cumulus cells and intracellular localization of NOS isoforms in the oocytes during meiotic maturation

All three NOS isoforms (eNOS, iNOS and nNOS) were found in both porcine oocytes and their cumulus cells. The highest expression of eNOS and iNOS isoforms was detected in oocytes at the GV stage and it decreased during the course of meiotic maturation. The highest expression of nNOS was found at the MI stage (Fig. 1). The intracellular localization of NOS isoforms in the oocytes differed. eNOS protein was present in oocyte cytoplasm as dots. In oocytes at the GV stage, the dots were concentrated especially in the germinal vesicles and subcortical regions. In oocytes at the MI and MII stages, the dots were dispersed in the cytoplasm and a higher level of the signal was also observed

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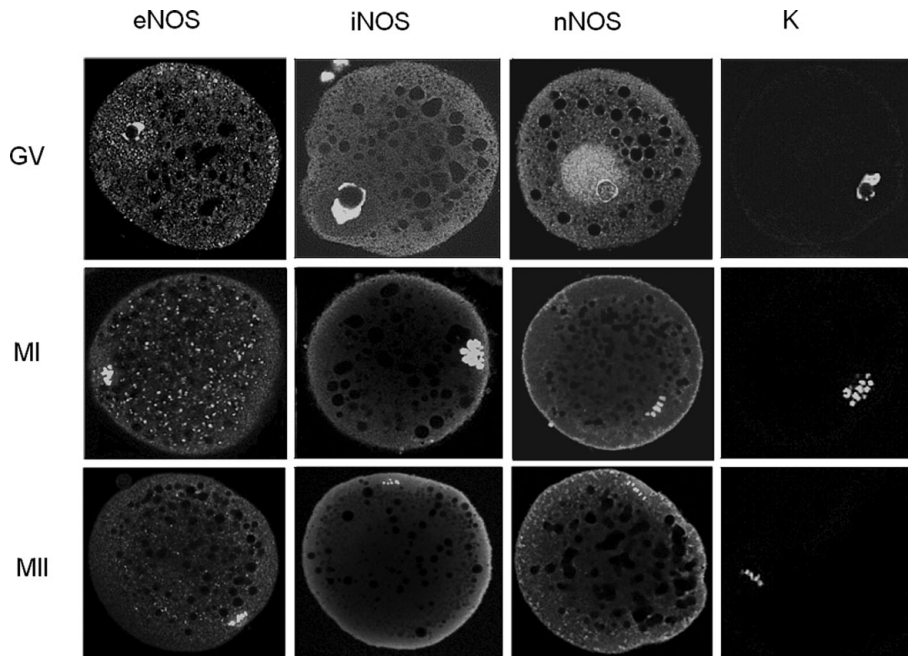

Figure 2 Subcellular localization of NOS isoforms in porcine oocytes. The oocytes were stained with rabbit anti-eNOS, anti-iNOS and anti-nNOS as first antibodies and anti-rabbit IgG–FITC as second antibody. DNA was stained by Hoechst 33258. Representative pictures of NOS localization are shown. GV – oocytes at germinal vesicle stage (0 h of culture), MI–oocytes at MI stage (24 h of culture), MII – oocytes at MII stage (48 h of culture), K – control oocytes exposed only to the second antibody. Magnification ×40.

in the region of meiotic spindle. iNOS protein was evenly dispersed in the oocyte cytoplasm. The highest intensity of the signal was recorded at the GV stage and gradually decreased at the MI and MII stages. In contrast to eNOS, iNOS protein was not concentrated either in the germinal vesicle or in the region of meiotic spindle. nNOS protein was localized in the oocyte cytoplasm at the GV stage and it accumulated the same as eNOS in the germinal vesicle. The signal in the cortical region increased during the course of meiotic maturation, but no higher signal associated with meiotic spindle was observed (Fig. 2). NOS mRNA of all three isoforms occurred in the porcine oocytes at the GV, MI and MII stages. While eNOS mRNA and iNOS mRNA were also found in cumulus cells, nNOS mRNA was not detected (Fig. 3).

Experiment 2. The effect of L-*NAME on meiotic maturation of pig oocytes*

The presence of L-NAME inhibitor in the culture medium inhibited the meiotic maturation of porcine oocytes in a dose-dependent manner. The number of oocytes that completed meiotic maturation significantly decreased. In the presence of 2.5 mM L-NAME, 25.5% of the oocytes were unable to complete their meiotic maturation. This concentration suppressed MI–MII transition. In the presence of 10.0 mM L-NAME, 75.1% of the oocytes were unable to reach the MII stage (Table 1) after 48 h of culture and the percentage of oocytes in other stages of meiotic maturation increased (data not shown). The highest concentration of L-NAME also suppressed GVBD of the oocytes.

Experiment 3. The effect of AG on meiotic maturation of porcine oocytes

Meiotic maturation was also affected by the presence of AG, a specific inhibitor of the iNOS isoform, in the culture medium. The inhibitor significantly suppressed meiotic maturation in a dose-dependent manner. At the inhibitor's highest concentration (10.0 mM), only 2.3% of the oocytes completed their meiotic maturation and 60.8% of the oocytes did not even resume meiotic maturation and remained at the GV stage (Table 2). After removal of the inhibitor, the majority of the oocytes continued with meiotic maturation.

Experiment 4. The effect of NOS inhibition on GVBD and MI–MII transition and the role of cumulus cells in GVBD of oocytes influenced by NOS inhibitors

Both NOS inhibitors significantly suppressed GVBD, but AG was significantly more effective. After 24 h of culture with L-NAME, 48.2% of the oocytes reached the MI stage, in the presence of AG only 5.1% reached this stage (Table 3) however the MI–MII transition was suppressed by both inhibitors to the same extent. In the

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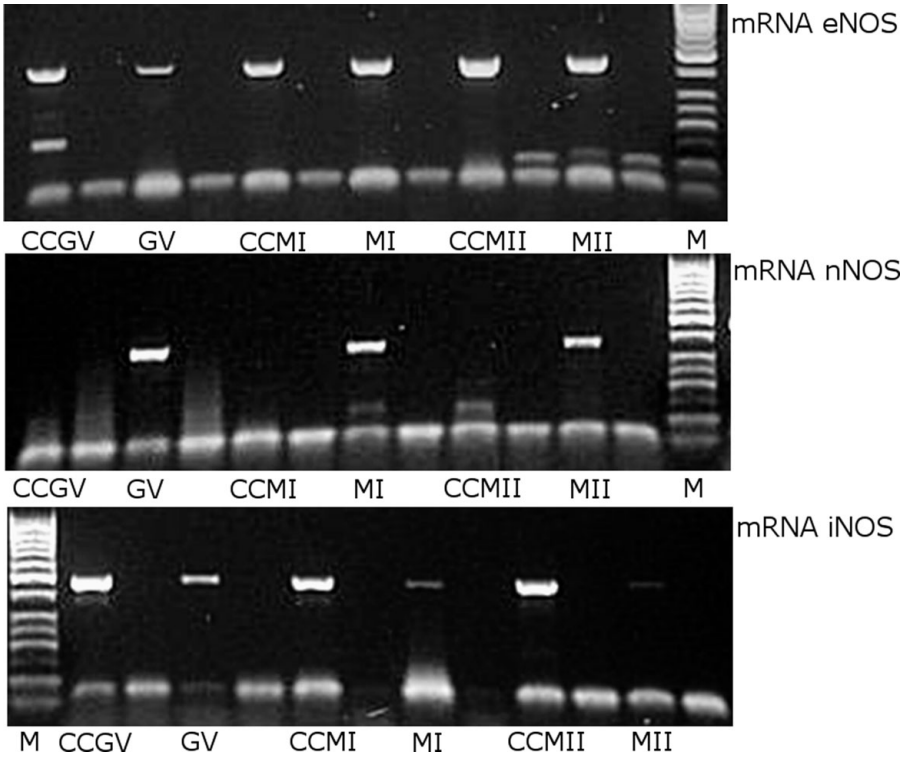

Figure 3 NOS mRNAs in porcine oocytes and their cumulus cells. Oocytes were cultured for 0, 24 and 48 h. RNA was isolated from 50 oocytes and their cumulus cells, separately. The PCR products were separated using agarose gel electrophoresis and then visualized using ethidium bromide staining. GV, MI, MII – oocytes culture for 0, 24 and 48 h; CCGV, CCMI, CCMII – cumulus cells of oocytes cultured 0, 24 and 48 h; M, marker.

Table 1 Effect of the inhibitor L-NAME on the meiotic maturation of porcine oocytes.

		Stage of meiotic maturation(% ± SE)			
		GV	MI	MII	*n*
C		0.3 ± 0.1^{a}	12.3 ± 0.3^{a}	86.6 ± 0.4^{a}	120
L-NAME (mM)	1.0	0.0 ± 0.0^{a}	$16.8 \pm 1.1^{a,b}$	70.6 ± 0.7^{b}	121
	2.5	2.6 ± 0.3^{a}	25.5 ± 0.1^{b}	$63.3 \pm 0.5^{b,c}$	192
	5.0	2.5 ± 0.4^{a}	34.9 ± 0.9^{b}	47.1 ± 1.2^{c}	148
	10.0	16.4 ± 0.5^{b}	33.5 ± 1.1^{b}	24.9 ± 0.6^{d}	161
D-NAME (mM)	10.0	1.6 ± 0.2^{a}	9.5 ± 0.6^{a}	85.3 ± 0.5^{a}	132

$^{a-d}$Statistically significant differences between experimental and control groups of oocytes after different treatments with the L-NAME inhibitor (within the column) ($p < 0.05$). The same letters indicate statistical insignificance.
Oocytes were cultured in medium with NOS inhibitor (L-NAME), ineffective form of inhibitor (D-NAME) or in inhibitor free medium (C) for 48 h. GV, MI and MII – oocytes at the germinal vesicle stage, metaphase I and metaphase II respectively; *n*, number of oocytes in group.

presence of L-NAME in the culture medium, 51.5% of all oocytes reached the MII stage and, in the presence of AG, 57.5% of the oocytes reached the MII stage and excluded the first polar body (Table 3).

The effect was found with DO. The effective concentration of AG (10.0 mM) significantly increased the number of oocytes that were unable to resume meiosis (34%) and remained at the GVBD stage after 24 h of culture. On the other hand, the same L-NAME concentration that effectively inhibited the meiotic maturation of COC not only did not influence GVBD of DO, but also did not significantly decrease the percentage of oocytes maturing to the MI stage (Table 3).

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Table 2 Effect of the inhibitor aminoguanidine on the meiotic maturation of porcine oocytes.

		Stage of meiotic maturation (% ± SE)			
		GV	MI	MII	*n*
C		1.2 ± 0.3^{a}	8.1 ± 0.5^{a}	86.6 ± 0.4^{a}	125
AG (mM)	2.5	0.0 ± 0.0^{a}	$15.4 \pm 0.5^{a,b}$	59.3 ± 0.5^{b}	159
	5.0	1.2 ± 0.1^{a}	$9.4 \pm 0.5^{a,b}$	49.4 ± 0.3^{c}	153
	7.5	36.2 ± 0.4^{b}	$8.8 \pm 0.3^{a,b}$	22.4 ± 0.3^{d}	137
	10.0	60.8 ± 0.3^{c}	3.0 ± 0.3^{b}	2.8 ± 0.1^{e}	208

[a–e]Statistically significant differences between experimental and control groups of oocytes after different treatments with the aminoguanidine (within the column) ($p < 0.05$). The same letters indicate statistical insignificance.
Oocytes were cultured in medium with aminoguanidine (AG), or in inhibitor-free medium (C) for 48 h. GV, MI and MII – oocytes at the germinal vesicle stage, metaphase I and metaphase II respectively; *n*, number of oocytes in group.

Experiment 5. NO formation in COC during meiotic maturation and the effect of AG on NO formation

The quantity of NO metabolites, nitrates and nitrites significantly increased during meiotic maturation in COC. This finding means that NO was newly formed in COC. However, during the cultivation of COC in the presence of AG, no significant increase of NO metabolites was recorded (Fig. 4).

Discussion

In our experiments, the presence of eNOS, iNOS and nNOS isoforms was found in porcine oocytes and their cumulus cells. NOS mRNA of all three isoforms was also detected in oocytes and their cumulus cells with the exception of nNOS mRNA, which was not present in cumulus cells. eNOS and iNOS have been reported to occur in the oocytes of mice (Mitchel *et al.*, 2004), rats (Van Voorhis *et al.*, 1995; Jablonka-Shariff & Olson, 1997) and pigs (Hattori *et al.*, 2000; Takesue *et al.*, 2003; Tao *et al.*, 2004, 2005). We also detected the presence of nNOS and nNOS mRNA in porcine oocytes. nNOS mRNA was found in mouse oocytes (Abe *et al.*, 1999) and cattle oocytes (Tesfaye *et al.*, 2006), but the presence of nNOS protein has not yet been reported. The absence of nNOS mRNA in the cumulus cells is surprising because nNOS protein is present in them. It is possible that the nNOS protein might be synthesized in the cumulus cell and transported to the oocyte, because there is communication between the oocytes and the cumulus cells. However, the transport is mediated by gap junctions carrying only small molecules up to 1 kDa. Another explanation could be alternative splicing of nNOS mRNA. This causes the formation of a larger number of molecular species of nNOS mRNA and the formation of nNOS proteins that differ both in

Table 3 Effect of the inhibitors L-NAME and aminoguanidine (AG) on GVBD of oocytes with and without cumulus cells and first polar body exclusion.

		Stage of meiotic maturation(% ± SE)			
		GV	MI	MII	*n*
COC 24 h	C	1.6 ± 0.5^{a}	87.0 ± 0.5^{a}	–	120
	L-NAME (mM) 10.0	12.9 ± 0.4^{b}	48.2 ± 2.3^{a}	–	157
	D-NAME (mM) 10.0	1.4 ± 0.6^{a}	85.6 ± 0.5^{a}	–	131
	AG (mM) 10.0	65.0 ± 1.1^{c}	5.1 ± 0.8^{b}	–	129
DO 24 h	C	14.6 ± 0.2^{a}	52.8 ± 1.0^{a}	–	130
	L-NAME (mM) 10.0	16.2 ± 0.5^{a}	41.7 ± 1.9^{a}	–	121
	D-NAME (mM) 10.0	13.0 ± 0.5^{a}	48.1 ± 1.3^{a}	–	125
	AG (mM) 10.0	34.0 ± 0.6^{b}	36.0 ± 1.5^{a}	–	136
COC24 h+24 h	C	–	–	87.0 ± 0.9^{a}	128
	L-NAME (mM) 10.0	–	–	51.5 ± 1.6^{b}	164
	D-NAME (mM) 10.0	–	–	87.5 ± 0.4^{a}	132
	AG (mM) 10.0	–	–	57.5 ± 1.1^{b}	201

[a–c]Statistically significant differences between experimental and control groups of oocytes after different treatments with the L-NAME inhibitor (within the column) ($p < 0.05$). The same letters indicate statistical insignificance.
For determination of the effect on GVBD, the oocytes were cultured in medium with NOS inhibitors (L-NAME, AG), ineffective form of inhibitor (D-NAME), or in inhibitor free medium (C). Cumulus-cell-enclosed oocytes (COC 24 h) and denuded oocytes (DO 24 h) were cultured for 24 h.
For determination of the effect on first polar body exclusion, the cumulus-cell-enclosed oocytes were cultured for 24 h in inhibitor free medium and then 24 h medium with inhibitor (COC 24 + 24 h)
GV, MI and MII–oocytes at the GV stage, metaphase I and metaphase II respectively; *n*, number of oocytes in group.

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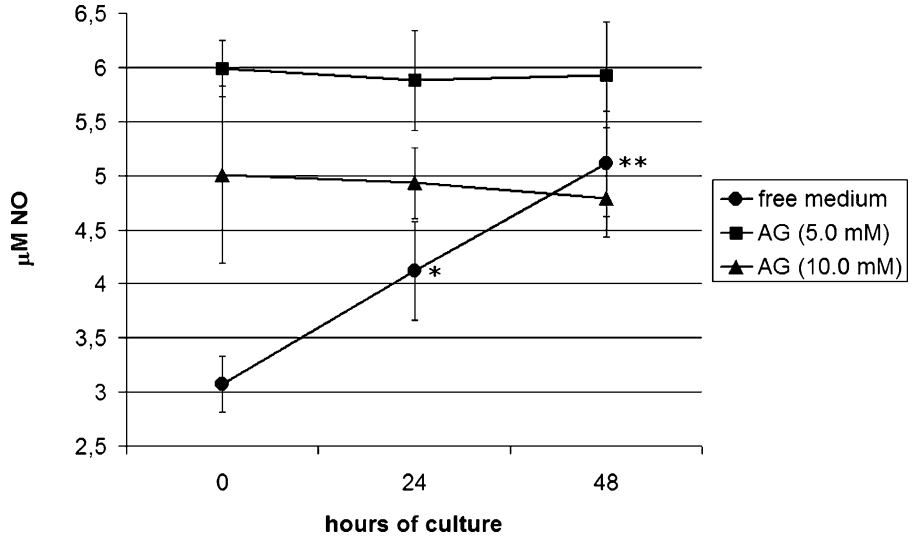

Figure 4 NO formation in porcine cumulus cell–oocyte complexes (COC) during *in vitro* meiotic maturation. COC (60 COC in each group) were cultured for 0, 24 and 48 h in inhibitor-free medium or in the medium with aminoguanidine (AG). Nitric oxide was determined by colorimetric measurement of NO nitric oxide metabolites, nitrites and nitrates. The concentration of NO was calculated on the base of calibration curve. *,** Statistically significant differences groups of COC (0, 24 and 48 h of culture), $p < 0.05$.

enzymatic activity and structure (Brenman *et al.*, 1997; Gonzales-Cadavid *et al.*, 2000). We can only speculate that nNOS mRNA was not detected by our method due to alternative splicing, as nNOS protein was detected by our antibodies.

NOS activity is necessary for the meiotic maturation of the oocyte (Jablonka-Shariff & Olson, 1998; Hattori *et al.*, 2000; Bu *et al.*, 2003; Tao *et al.*, 2004, 2005). As was also confirmed by our experiments, NOS is important for the meiotic maturation of porcine oocytes as the presence of both NOS inhibitors, L-NAME and AG, in the culture medium markedly influenced meiotic maturation in a dose-dependent manner. However, their effects are different.

L-NAME, as a non-specific NOS inhibitor, suppressed MI–MII transition. This effect has also been described in rat (Jablonka-Shariff & Olson, 1997) and mouse (Bu *et al.*, 2003) oocytes. At the same time, this concentration increased the percentage of porcine oocytes, similar to that in mouse oocytes (Jablonka–Shariff & Olson, 2000), in other stages of meiotic maturation. It was reported that L-NAME did not affect GVBD. However, in our experiments the highest concentration (10.0 mM) of L-NAME suppressed the GVBD of porcine oocytes as well. Despite L-NAME being considered an inhibitor of all NOS isoforms, it is more effective for eNOS (Lamas *et al.*, 1992). In our experiment, the eNOS isoform as well as the nNOS isoform was localized in the GV. In contrast to mouse oocyte (Huo *et al.*, 2005) we did not find the iNOS isoform in GV of porcine oocyte. On the other hand, only eNOS was associated with meiotic spindles. Su *et al.* (2005) have reported that eNOS colocalizes with microtubules in somatic cells. In contrast to mouse oocyte (Huo *et al.*, 2005) we did not find the iNOS isoform in GV and around the spindle. Therefore, we can assume that eNOS and nNOS activity is important for GVBD and that eNOS activity is important for the correct function of the spindle of porcine oocytes. However, further experiments are necessary for precise clarification of the roles of eNOS and nNOS in GVBD and meiotic spindle function.

The highest concentration (10.0 mM) of AG, a specific inhibitor of iNOS, effectively suppressed GVBD of porcine oocytes in a similar way to L-NAME. A similar effect of the same AG concentration was also described in cattle oocytes by Bilodeau-Goeseels (2007), although Matta *et al.* (2008) reported that this concentration did not affect meiotic maturation of cattle oocytes. Instead, they found 100 mM to be effective. In our experiment, the GVBD of porcine oocytes was inhibited by AG more effectively than by L-NAME and the majority of oocytes did not undergo meiosis, in contrast to L-NAME. iNOS protein, unlike eNOS and nNOS protein, was not localized in the GV, but was evenly dispersed in the cytoplasm. The concentration of iNOS protein was the highest in oocytes in the GV stage. On the other hand, AG in our experiments also inhibited MI–MII stage transition when it was added to the culture medium of oocytes after a previous 24-h culture in the inhibitor-free medium. This fact suggests that iNOS activity is significant not only for GVBD, but also for the further progression of meiotic maturation.

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The different effects of AG and L-NAME on porcine oocytes were also found in denuded oocytes. AG inhibited the GVBD of COC, as well as the GVBD of DO, even more effectively. Tao *et al.* (2005) reported that NO formed by iNOS in cumulus cells was necessary not only for their expansion, but also for the meiotic maturation of the oocytes. The enhancement of the inhibitory effect of aminoguanidine on GVBD may also be due to the fact that penetration of the inhibitor is easier in denuded oocytes because of the absence of the cumulus-cell barrier. On the basis of our experiments, it can be assumed that the meiotic maturation of pig oocytes is significantly influenced not only by iNOS activity in cumulus cells, but also in oocyte cytoplasm. On the other hand, although the competitive NOS inhibitor L-NAME significantly affects GVBD of oocytes with cumulus cells, it has no significant effect on GVBD of denuded oocytes. These results might indicate that the role of NOS in cumulus cells is more important for meiotic maturation than it is in oocyte cytoplasm. A similar effect was also reported following NOS inhibition by L-NAME in mouse oocytes (Bu *et al.*, 2003). However, L-NAME and AG differ in their mechanism of NOS inhibition. In contrast to L-NAME, which is a competitive inhibitor, the inhibitory mechanism of AG is based on covalent modification of the enzyme and, although AG is reported as an iNOS specific inhibitor, it also inhibits to some extent eNOS and nNOS (Alderton *et al.*, 2001). This seems to be the reason why the effect of L-NAME on GVBD is not so marked as in the case of AG. The effect of L-NAME is probably disguised by the removal of cumulus cells, which play an important role in GVBD (Wongsrikeao *et al.*, 2005).

We found that NO in COC was produced during the course of culture. The presence of AG in the culture medium significantly suppressed the formation of NO by COC. This effect of NOS inhibition was also described by Hattori *et al.* (2004), but the authors considered the eNOS isoform to be responsible for NO production. As it is evident from our experiments, AG as an iNOS inhibitor affects NO production. However, as was mentioned above, AG also partially inhibits eNOS and nNOS isoforms (Alderton *et al.*, 2001). Therefore, it is not possible to conclude that only iNOS is responsible for NO formation during meiotic maturation and it is probable that iNOS together with eNOS, or all three isoforms are involved.

On the basis of our experiments, it can be concluded that all the three NOS isoforms are present in porcine oocytes and their activity is essential for the course of meiotic maturation. During this process, NO is formed in COC and NO formed by cumulus cells is necessary for the GVBD.

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