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Inducible nitric oxide synthase-derived nitric oxide regulates germinal vesicle breakdown and first polar body emission in the mouse oocyte

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Abstract

The present study investigated the subcellular localization of inducible nitric oxide synthase (iNOS) during mouse oocyte meiotic maturation and fertilization using confocal microscopy, and further studied the roles of iNOS-derived NO in oocyte maturation by using an iNOS-specific inhibitor aminoguanidine (AG) and iNOS antibody microinjection. In germinal vesicle-stage oocytes, iNOS immunoreactivity was mainly localized in the germinal vesicle. Shortly after germinal vesicle breakdown, the iNOS immunoreactivity accumulated around the condensed chromosomes. At metaphase I and metaphase II, with the organization of chromosomes to the equatorial plate, iNOS immunoreactivity was concentrated around the aligned chromosomes, putatively the position of the metaphase spindle. The accumulation of iNOS immunoreactivity could not be detected at anaphase I and anaphase II. However, at telophase I and telophase II, the staining of iNOS was concentrated in the region between the separating chromosomes/chromatids. Furthermore, the staining of iNOS also accumulated in the male and female pronuclei in fertilized eggs. Germinal vesicle breakdown and the first polar body emission of the oocytes were significantly blocked by the iNOS-specific inhibitor AG in a dose-dependent manner. The germinal vesicle breakdown in oocytes injected with iNOS antibody was also inhibited. We found that the phosphorylation of mitogen-activated protein kinase in oocytes after germinal vesicle breakdown was inhibited by AG treatment. The control oocytes extruded a normal first polar body, while the AG-treated oocytes exhibited an elongated protrusion or no elongated protrusion. The results of confocal microscopy showed that the AG-treated oocytes were arrested at anaphase I–telophase I. Our results suggest that the iNOS-derived NO pathway plays important roles in mouse oocyte meiotic maturation, especially in germinal vesicle breakdown and the anaphase–telophase transition.

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Introduction

Fully-grown mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase, which is also termed the germinal vesicle (GV) stage. GV-stage-arrested oocytes can spontaneously resume meiosis when they are released from the inhibitory environment of follicles. However, these oocytes arrest again at the metaphase of meiosis II. With the separation of sister chromatids and emission of the second polar body triggered by fertilization, meiosis is completed (Josefsberg *et al.* 2001). Nitric oxide (NO), a cell messenger, is formed from L-arginine by isoforms of NO synthases (NOSs) via NG-hydroxy-L-arginine, with L-citrulline as a by-product. Isoforms of NOSs are composed of neuronal NOS, endothelial NOS (eNOS) and inducible NOS (iNOS). NO plays multiple roles in different biological systems, and it is implicated in the control of follicle and oocyte function (Mitchell *et al.* 2004). It has been reported that iNOS or eNOS could be detected in porcine or mouse follicles, granulosa cells, cumulus cells and oocytes (Hattori *et al.* 2001, Takesue *et al.* 2003, Mitchell *et al.* 2004). Expression of eNOS increases after the luteinizing hormone surge or human chorionic gonadotropin (hCG) injection (Van Voorhis *et al.* 1995, Jablonka-Shariff & Olson 1998, Nakamura *et al.* 1999), and eNOS-derived NO stimulates the ovulatory process (Shukovski & Tsafriri 1994, Powers *et al.* 1995,

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Bonello *et al.* 1996, Hesla *et al.* 1997, Yamauchi *et al.* 1997, Jablonka-Shariff & Olson 1998, Jablonka-Shariff *et al.* 1999). The reports about changes in iNOS expression during this process are controversial (Matsumi *et al.* 1998), and the role of iNOS-derived NO is unclear. NOS inhibitors, N-omega-nitro-L-arginine methyl ester (L-NAME), N-omega-nitro-L-arginine (L-NNA) or aminoguanidine (AG) bicarbonate salt affected the process of oocyte meiotic maturation, including inhibition of GV breakdown (GVBD) and the first polar body (PB1) emission in *in vitro* cultured pig, mouse or rat oocytes (Blashkiv *et al.* 2001, Bu *et al.* 2003, Voznesens'ka & Blashkiv 2003, Tao *et al.* 2004). The results from experiments using NO donors also suggest that NO may have crucial roles in oocyte maturation, such as GVBD (Sengoku *et al.* 2001, Voznesens'ka & Blashkiv 2003). On the other hand, others reported that AG promotes GVBD in mouse preovulatory follicles, and S-nitroso-L-acetyl penicillamine (SNAP), an NO donor, prevented this effect; SNAP dose-dependently inhibited GVBD in denuded oocytes (DOs) (Nakamura *et al.* 2002). But it has also been found that NO has dual functions (stimulation or inhibition) in mouse meiotic maturation depending on its concentration (Bu *et al.* 2003). Overall, the exact roles of NO in oocyte maturation remain unclear and need to be further studied. In this study, we for the first time revealed the subcellular localization of iNOS at different stages of mouse oocyte meiotic maturation and fertilization by confocal microscopy. Since the reports on the role of NO in GVBD are still controversial and since it is still not clear at what stage the NO acts to affect the meiotic cell cycle after GVBD, we further investigated the roles of iNOS-derived NO in mouse oocyte maturation by using the iNOS-specific inhibitor, AG, and iNOS antibody microinjection. Our findings show that iNOS-derived NO is required for GVBD and anaphase–telophase transition.

Materials and Methods

Chemicals and solutions

Polyclonal rabbit anti-murine iNOS antibody and the iNOS-specific inhibitor AG were purchased from Cayman Chemical Company (Ann Arbor MI, USA). AG was diluted just before use. All other chemicals or components of media were embryo culture or cell culture grade and were obtained from Sigma unless otherwise noted.

Mouse oocyte and zygote collection

Kunming mice, a native breed widely used in biological research in China, were used for oocyte and zygote collection. Animal care and handling were conducted in accordance with policies promulgated by the ethical committee of the Institute of Zoology, Chinese Academy of Sciences. Both denuded and cumulus-enclosed GV-intact oocytes were obtained as previously described by Tong *et al.* (2002) and maintained in M2 medium supplemented with 60 μg/ml penicillin and 50 μg/ml streptomycin, and cultured in M2 medium. All cultures were carried out at 37 °C in a humidified atmosphere of 5% CO_2. The oocytes at different stages were collected for confocal microscopy. For the collection of metaphase II-arrested eggs, females were superovulated by i.p. injection of 10 IU pregnant mare serum gonadotropin followed 46–48 h later with 10 IU hCG. Mice were killed and oviducts were removed at 14–16 h after hCG injection. Using a pair of fine forceps to tear the oviducts, cumulus masses were collected in M2 medium. To remove the cumulus cells, eggs were briefly exposed to 300 IU/ml hyaluronidase followed by three washes in M2 medium. *In vitro* fertilization was performed using 1×10^6/ml motile cauda epididymal sperm, which had been previously capacitated in M16 medium with 2.5 mM taurine for 1 h. The fertilized eggs were collected at different stages for confocal microscopy.

Confocal microscopy of mouse oocytes

After removal of Zona Pellucida in acidified Tyrode's solution (pH 2.5), oocytes or embryos at the desired stages were fixed in 4% paraformaldehyde in PBS for 30 min, permeabilized for 30 min in the incubation buffer (0.5% Triton X-100 in 20 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose, 0.02% NaN_3), then washed in PBS with 0.1% Tween 20 three times, and finally incubated with polyclonal rabbit anti-human iNOS antibody diluted 1:100 for 1 h. The oocytes or fertilized eggs were rinsed three times and incubated for 1 h with 1:100 FITC-conjugated goat anti-rabbit IgG, followed by three washes and staining with 10 μg/ml propidium iodide. Finally, the oocytes or fertilized eggs were added to a glass slide, mounted in 1,4-diazabicyclo(2.2.2)octane hydrochloride-containing medium, and covered with a coverglass. The samples were examined using a TCS-4D laser scanning confocal microscope (Leica Microsystems, Bensheim, Germany). As a negative control, the first antibody was replaced by rabbit IgG.

Antibody microinjection

Polyclonal rabbit anti-human iNOS antibody (100 μg/ml in PBS) was injected into the GV-arrested oocytes as described by Tong *et al.* (2002). An Eppendoff microinjector was used in this experiment. All microinjections were performed by using a beveled micropipette to minimize damage and were finished within 30 min. A microinjection volume of about 7 pl per oocyte was used in all experiments. Each experiment consisted of three separate groups and approximately 50 oocytes were injected in each group. The same amount of rabbit IgG was injected into the oocytes as the negative control. 3-Isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, was always included at 4 mM in M2 medium to prevent oocyte GVBD during the process of antibody microinjection. After

microinjection of iNOS antibody, the oocytes were thoroughly washed in M2 medium and then cultured for 2 h, and then the GVBD was examined.

Western blot analysis

For detection of active ERK1/2, proteins from 50 oocytes were collected in SDS sample buffer and heated to 100 °C for 4 min. After cooling on ice and centrifuging at 12 000 *g* for 3 min, samples were frozen at −20 °C until use. The total proteins were separated by SDS-PAGE with a 4% stacking gel and a 10% separating gel for 30 min at 90 V and 2.5 h at 120 V respectively, and then electrophoretically transferred onto nitrocellulose membrane for 2.5 h at 200 mA, at 4 °C. Then the membrane was blocked overnight at 4 °C in TBST buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% low-fat milk. After that, the membrane was incubated for 2 h at 37 °C in TBST with 1:500 mouse anti-human p-ERK1/2 antibody. After three washes of 10 min each in TBST, the membrane was incubated for 1 h at 37 °C with horseradish peroxidase-conjugated rabbit anti-mouse IgG diluted 1:1000 in TBST. The membranes were washed three times in TBST and then processed using an enhanced chemiluminescence detection system (Amersham). All experiments were repeated at least three times.

Statistical analysis

All percentages from three repeated experiments are expressed as means ± S.E.M. and the numbers of oocytes observed are labeled in brackets as ($n =$). All frequencies were subjected to arcsin transformation. The transformed data were statistically compared by ANOVA using SPSS software followed by Student–Newman–Keuls test. Differences at $P < 0.05$ were considered to be statistically significant.

Experimental design

Experiment 1

The subcellular localization of iNOS during normal oocyte meiotic maturation and fertilization was examined by confocal microscopy.

Experiment 2

To study the role of iNOS-derived NO during oocyte GVBD, fully grown GV-intact oocytes were collected and cultured in M2 medium containing 0, 1, 10 or 50 mM iNOS-specific inhibitor AG for 14 h. At the end of culture, the oocytes were examined for GV integrity or collected for Western blotting. Furthermore, the GV-stage-arrested oocytes were microinjected with iNOS antibody and then cultured for 2 h, and the GVBD was examined at the end of the culture.

Experiment 3

To study the possible roles of iNOS-derived NO during the PB1 emission, fully grown GV-intact oocytes were collected and cultured in M2 medium for 4 h and then the oocytes that had undergone GVBD were collected and cultured in M2 medium containing 0, 1 or 10 mM AG for an additional 12 h. At the end of culture, the oocytes were examined for PB1 emission or collected for confocal microscopy.

Results

Subcellular localization of iNOS during oocytes meiotic maturation and fertilization

The subcellular localization of iNOS during mouse oocyte meiotic maturation and fertilization is shown in Fig. 1. The specimens were stained with propidium iodide to visualize the DNA and confirm the stage of meiotic maturation. The localization of iNOS varied at different developmental stages. In GV-stage oocytes, iNOS immunoreactivity was mainly localized in the GV (Fig. 1A). Shortly after GVBD, iNOS immunoreactivity accumulated around the condensed chromosomes (Fig. 1B). At metaphase I (Fig. 1C) and metaphase II (Fig. 1F), with the organization of chromosomes to the equatorial plate, the immunoreactivity of iNOS was concentrated around the aligned chromosomes, putatively the position of the metaphase spindle. The accumulation of iNOS immunoreactivity in the spindle region could not be detected at anaphase I (Fig. 1D) and anaphase II (Fig. 1G); however, at telophase I (Fig. 3E) and telophase II (Fig. 1H), the staining of iNOS was concentrated in the region between the separating chromosomes. Furthermore, the staining of iNOS also accumulated in the male and female pronuclei in fertilized eggs (Fig. 1I).

Inhibition of iNOS-derived NO prevents oocyte GVBD

As shown in Fig. 2A, the GVBD of the DOs was significantly blocked by iNOS-specific inhibitor AG in a dose-dependent manner. The GVBD rate of cumulus-enclosed oocytes (CEOs) was 94% ($n = 160$), while the GVBD of CEOs was completely inhibited by 50 mM AG ($n = 112$). Furthermore, the phosphorylation of mitogen-activated protein kinase (MAPK) in the control oocytes could be detected at 14 h after culture in M2 medium, but was completely inhibited in 50 mM AG-treated oocytes (Fig. 2B). We also found that the GVBD of oocytes microinjected with iNOS antibody was evidently inhibited (33%, $n = 106$) compared with the control group (66%, $n = 120$).

iNOS-specific inhibitor blocks PB1 emission

As shown in Fig. 3A, the PB1 emission of oocytes was blocked by the iNOS-specific inhibitor AG in a dose-dependent manner. The PB1 emission rate of DOs was

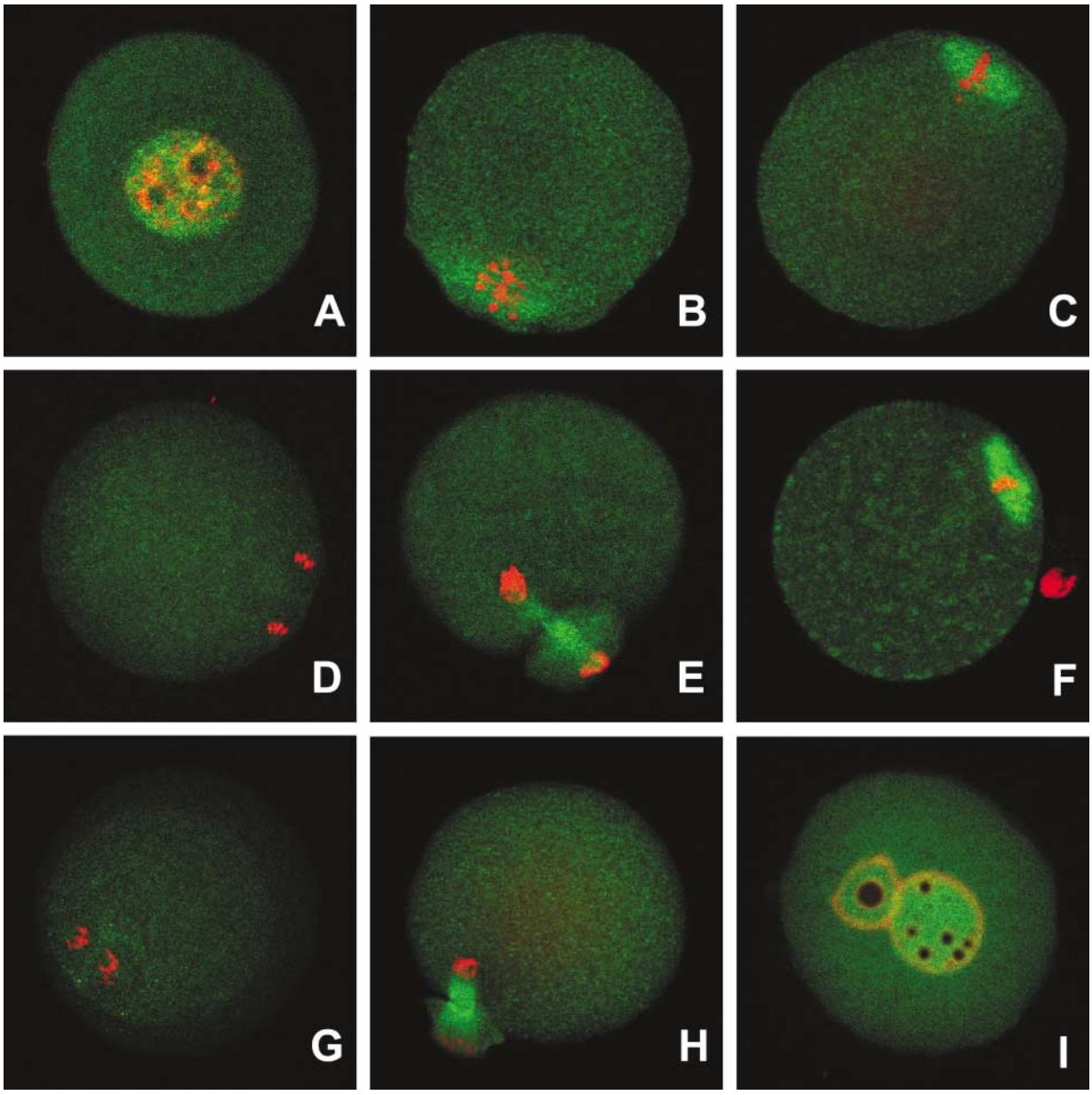
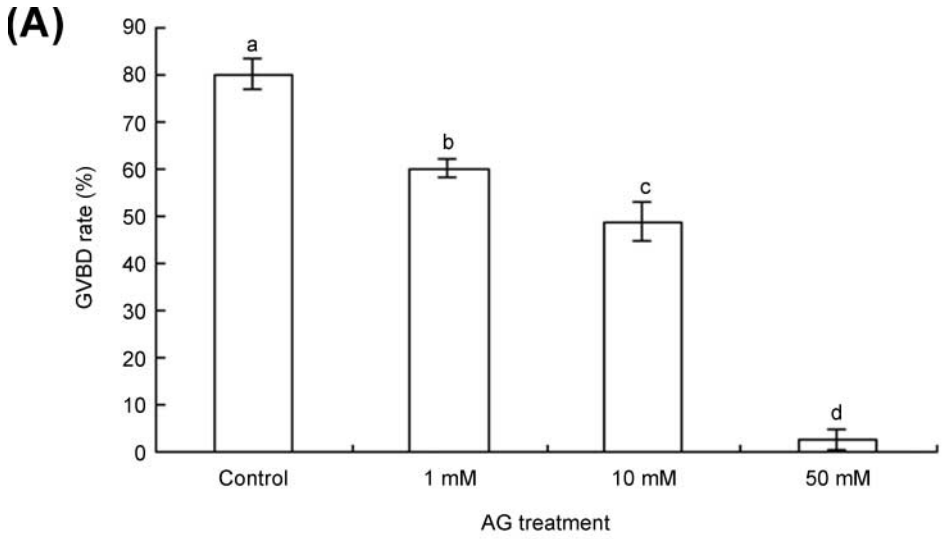


Figure 1 The distribution of iNOS during mouse oocyte meiotic maturation and fertilization. Red, DNA; green, iNOS. iNOS was mainly localized in the GV (A). Shortly after GVBD, iNOS accumulated around the condensed chromosomes (B). At metaphase I (C) and metaphase II (F), iNOS was concentrated around the aligned chromosomes, putatively the position of the metaphase spindle. The accumulation of iNOS could not be detected at anaphase I (D) and anaphase II (G); however, at telophase I (E) and telophase II (H), the staining of iNOS was concentrated in the region between the separating chromosomes/chromatids. iNOS accumulated in the male and female pronuclei in fertilized eggs (I).

78 ± 5% in the control group, but was only 37 ± 4% in 1 mM AG-treated oocytes, and PB1 emission was completely inhibited by 10 mM AG. The AG-treated oocytes exhibited an elongated protrusion or no elongated protrusion. The results of α-tubulin staining examined by confocal microscopy showed that the control oocytes were arrested at the metaphase II and a normal PB1 was extruded (Fig. 3Ba), while the AG-treated oocytes were arrested at anaphase I–telophase I transition (Fig. 3Bc and b).

Discussion

NO plays multiple roles in different biological systems, and it is implicated in the control of ovary and follicle function, such as follicle development, ovulation and ovarian steroidogenesis (Jablonka-Shariff *et al.* 1999, Mitchell *et al.* 2004). Although numerous experiments have revealed that the NO pathway affects ovarian function and follicle development (Hattori *et al.* 2001, Takesue *et al.* 2003, Mitchell *et al.* 2004), most results are derived



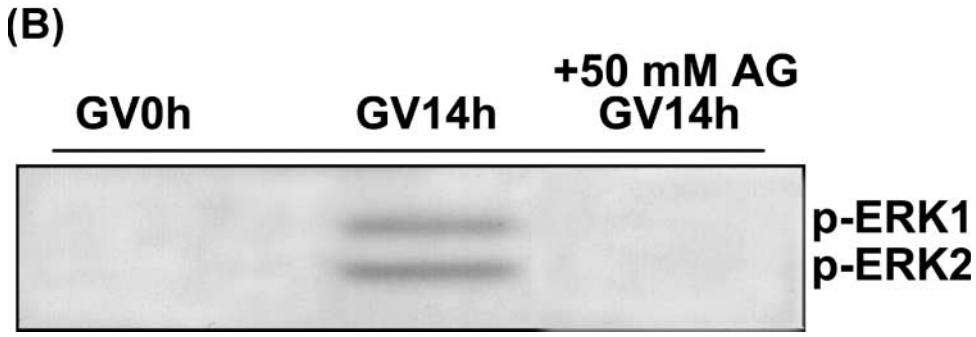
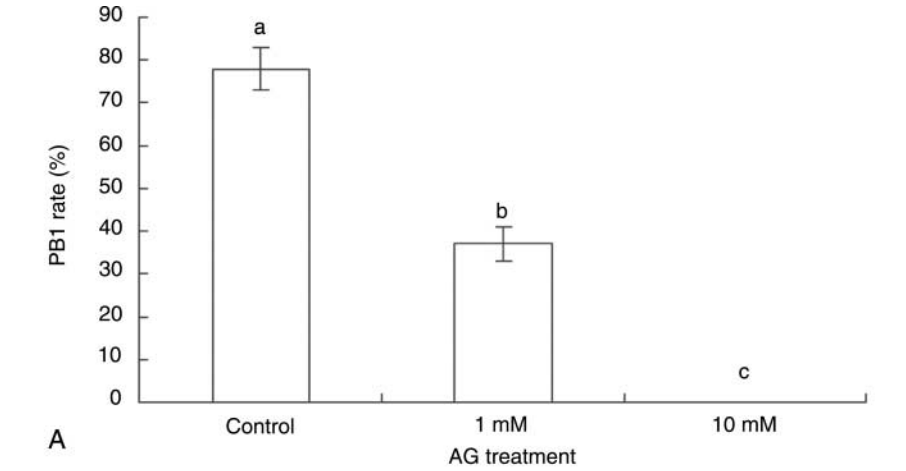


Figure 2 Inhibition of iNOS-derived NO prevents oocyte GVBD and MAPK phosphorylation. GVBD was observed 14 h after culture. The GVBD of the DOs was significantly inhibited by the iNOS-specific inhibitor AG in a dose-dependent manner (A). Means ± S.E.M. Letters a, b, c, d above columns denote significant differences at $P < 0.05$. Phosphorylation of MAPK in control oocytes was significantly higher than that in AG-treated oocytes (B).



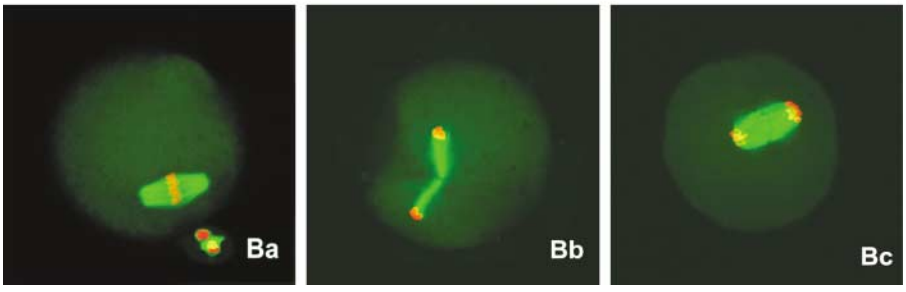


Figure 3 iNOS-specific inhibitor blocks PB1 emission. GV-arrested oocytes were cultured in M2 medium with different concentrations of AG for 12 h. At the end of culture, the oocytes were examined for PB1 emission (A) or collected for confocal microscopy (B). The PB1 emission of oocytes was blocked by the iNOS-specific inhibitor AG in a dose-dependent manner (A). Means± S.E.M. Letters a, b, c above columns denote significant differences at $P < 0.05$. The AG-treated oocytes were arrested at anaphase I–telophase I transition (Bb and Bc). The control oocytes were arrested at metaphase II (Ba).

from the follicles or the interaction between the oocyte and granulosa cells, while whether NO acts directly on oocytes, especially what function iNOS-derived NO plays during oocyte meiosis, is unclear (Bu *et al.* 2003, Tao *et al.* 2004). In this study, we for the first time revealed the subcellular localization of iNOS at different stages of mouse oocyte meiotic maturation and fertilization using confocal microscopy, and by using the iNOS-specific inhibitor AG and antibody microinjection, we studied the roles and the possible mechanisms of iNOS-derived NO in GVBD and PB1 emission. Our results show that iNOS has a specific subcellular localization throughout oocyte maturation. The process of GVBD and the PB1 emission of oocytes was significantly inhibited by iNOS-specific inhibitor AG in a dose-dependent manner. The phosphorylation of MAPK was inhibited by AG and the PB1 emission process was arrested at the anaphase–telophase transition. Our results also show that the GVBD of oocytes injected with iNOS antibody was inhibited compared with the control group. Our results suggest that the iNOS-derived NO pathway plays a crucial role in mouse oocyte meiotic maturation, especially in GVBD and the anaphase–telophase transition. It has been shown that eNOS is expressed in the porcine oocyte, granulosa cells and cumulus cells (Hattori *et al.* 2001, Takesue *et al.* 2003). iNOS and eNOS were localized in mouse ovaries, and omission of L-arginine significantly reduced follicle survival and ovulation (Nishikimi *et al.* 2001, Nemade *et al.* 2002, Mitchell *et al.* 2004, Tao *et al.* 2004). Furthermore, by using an NO donor and NO inhibitor, it has been proven that the NO pathway plays important roles in ovarian function and follicle development (Sengoku *et al.* 2001). The ovarian defects observed in eNOS knock-out mice suggest that eNOS-derived NO is a modulator of oocyte meiotic maturation (Jablonka-Shariff & Olson 2000). Our results showed that iNOS concentrated in the GV. The phosphorylation of MAPK was inhibited by AG. These results suggest that iNOS-derived NO affects the meiotic resumption of oocytes and signals/functions within the oocytes. Others also showed that AG markedly inhibited porcine oocyte meiotic resumption (Tao *et al.* 2004). Inhibitors of NOS also influence oocyte maturation in the rat (Jablonka-Shariff *et al.* 1999) and rabbit (Yamauchi *et al.* 1997). However, it has been found that nitrate/nitrite concentrations in preovulatory follicles significantly decrease after hCG injection and that iNOS plays a main role in the decrease of the intrafollicular NO concentration (Nakamura *et al.* 2002). Furthermore, both AG and hCG promoted oocyte GVBD in follicles cultured *in vitro*, and AG decreased intrafollicular cGMP levels (Nakamura *et al.* 2002). These results are different from our data. Nakamura *et al.* (2002) cultured the follicles, and iNOS–NO may play its roles through the cells in the follicle wall, while we cultured the oocytes, and iNOS–NO may play its roles through cumulus cells or oocytes. Furthermore, the intrafollicular components may also affect the function of iNOS–NO. Overall, we predict that the iNOS–NO–cGMP axis may play independent roles in oocyte maturation and follicular development. The accumulation of iNOS was observed in the midbody between the separating chromosomes/chromatids at telophase I and telophase II, suggesting that iNOS-derived NO may be involved in the polar body emission. Others also found that a NOS inhibitor (L-NAME) blocked the PB1 extrusion in porcine oocytes (Tao *et al.* 2004). Both NOS inhibitors (L-NAME and L-NNA) suppressed the PB1 emission in mouse CEOs in a dose-dependent manner (Bu *et al.* 2003). Moreover, previous studies showed that fewer oocytes from eNOS knock-out mice entered the metaphase of the second meiosis, and a greater percentage remained in metaphase I or were atypical relative to those in wild-type mice (Jablonka-Shariff & Olson 1998). Our results show that the process of PB1 emission is arrested at the anaphase–telophase transition by AG, so we predict that iNOS-derived NO is involved in the polar body emission by regulating the anaphase–telophase transition. In conclusion, our data demonstrate that iNOS-derived NO plays crucial roles within oocytes during meiotic maturation, especially in GVBD and PB1 emission.

Acknowledgements

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