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C-type natriuretic peptide inhibits porcine oocyte meiotic resumption

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Summary

C-type natriuretic peptide (CNP) is a recently identified meiotic inhibitor in mice. However, it has not been investigated in porcine oocytes to date. This study aimed to demonstrate the inhibitory effect of CNP against germinal vesicle breakdown (GVBD) in porcine oocyte meiotic resumption. Immunohistochemical analysis revealed intense natriuretic peptide receptor 2 (NPR2) immunoreactivity in the oocyte surrounded cumulus cells in the follicles. Furthermore, reverse transcription polymerase chain reaction (RT-PCR) analysis showed the expression of *npr2* mRNA only in cumulus cells but not in oocytes, suggesting that cumulus cells are the targets of CNP. When cumulus–oocyte complexes (COCs) or denuded oocytes (DOs) were cultured with various concentrations of CNP (10, 50, 100, 500, and 1,000 nM), inhibitory effect was observed in the COC group, but not in the DO group, confirming that CNP indirectly inhibits GVBD via cumulus cells. This evidence is the first indication that the CNP-NPR2 pathway is involved in meiotic arrest in porcine oocytes. Furthermore, we investigated the effect of oocyte-derived paracrine factor (ODPF) on *npr2* mRNA expression level in cumulus cells by evaluating changes in mRNA expression in oocytectomised COCs (OXCs) by real-time PCR. A significant decrease in *npr2* mRNA expression level was observed in OXCs, whereas mRNA expression level was restored in OXCs with DOs, indicating that ODPF participates in the regulation of *npr2* expression in porcine cumulus cells.

Keywords: C-type natriuretic peptide, Germinal vesicle breakdown, Natriuretic peptide receptor 2, Oocytectomy, Porcine

Introduction

In the mammalian reproductive system, oocytes are arrested within ovarian follicles at the diplotene stage of the first meiotic prophase; this is termed as meiotic arrest. During meiotic arrest, the intact nuclear membrane forms a germinal vesicle characteristic of this phase. Once signal transduction is triggered by gonadotropin in cumulus cells surrounding the oocyte, germinal vesicle breakdown (GVBD) is induced by the closing of gap junctions and a decrease in intra-oocyte cAMP concentration in mice and pigs (Webb *et al.*, 2002; Fan *et al.*, 2004). Intriguingly, as mammalian oocytes cultured *in vitro* undergo GVBD, it has been suggested that an inhibitor of meiotic resumption is present *in vivo* (Pincus & Enzmann, 1935). Indeed, hypoxanthine isolated from porcine follicular fluid prevents oocyte maturation (Downs *et al.*, 1985). Hypoxanthine has been also found in follicular fluids from other species (Kadam & Koide, 1990), and induction of meiotic arrest by hypoxanthine has been observed in a wide range of species (Eppig & Downs, 1987; Warikoo & Bavister, 1989; Gotze *et al.*, 1990); hypoxanthine is considered one of the active meiotic inhibitors in mammalian ovary.

C-type natriuretic peptide (CNP) belongs to the natriuretic peptide family, which comprises three similar but genetically distinct peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and CNP (Potter *et al*, 2006). These peptides have different affinities to their receptors. In contrast to ANP and BNP, which have high affinity to natriuretic

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peptide receptor 1 (NPR1), CNP has high affinity to natriuretic peptide receptor 2 (NPR2). The signature characteristic of natriuretic peptides is the production of cyclic guanosine monophosphate (cGMP), and their biological activities are mediated through a cGMP-dependent pathway. Several functions of natriuretic peptides have been reported, such as regulation of blood pressure (Woodard & Rosado, 2008) and natriuresis (Ballermann & Brenner, 1987).

Natriuretic peptides also play an important role in oocyte meiotic resumption. cGMP has been reported to be a crucial factor in maintaining meiotic arrest by entering into the oocyte through gap junctions and inhibiting the activity of oocyte phosphodiesterase 3, which hydrolyses cAMP in mice (Norris *et al.*, 2009). Törnell *et al.* (1990) have reported that ANP induces cGMP production in rat cumulus–oocyte complexes (COCs), and thereby inhibits spontaneous meiotic resumption. cGMP also acts as a GVBD inhibitor in porcine oocytes (Petr *et al.*, 2006), and the function of ANP as a meiotic inhibitor has also been reported. Recently, CNP has been reported to act as a meiotic inhibitor in mice (Zhang *et al.*, 2010). The report showed that cGMP produced in cumulus cells diffuses into oocytes through gap junctions and helps maintain the cAMP level in the oocytes, thereby preventing meiotic resumption. Histological analysis of *cnp*- and *npr2*-mutant mouse ovaries revealed that these models failed to maintain meiotic arrest, strongly suggesting the importance of the CNP-NPR2 pathway in oocyte maturation. However, the effect of CNP on porcine oocyte meiotic resumption was not determined. This study aims to demonstrate the effect of CNP on porcine oocyte meiotic resumption.

The present study also aims to investigate the regulation mechanism of CNP cognate receptor, *npr2*, in a porcine model. In the mouse model, *npr2* mRNA expression was regulated by oocyte-derived paracrine factors (ODPF). To demonstrate the effect of ODPF in the porcine model, we examined *npr2* mRNA expression in oocytectomised COCs (OXCs).

Materials and methods

Chemicals

The chemicals used in the study were obtained from Sigma-Aldrich unless stated otherwise.

Histological analysis of NPR2 localisation in porcine ovarian follicles

Porcine ovaries in prepubertal gilts were transected and fixed in a methacarn solution (methanol/chloroform/acetic acid, 6:3:1) for 48 h. After dehydration, the samples were embedded in paraffin and cut into 10-μm sections. The sections were kept until histological staining. For immunohistochemistry, deparaffinised sections were boiled for 25 min at 90°C to retrieve the antigen in HistoV (Nacalai Tesque, Kyoto, Japan). Then, the sections were treated with a blocking buffer (Blocking One; Nacalai Tesque) for 1 h at 4°C and with an antibody against NPR2 (Santa Cruz Biotechnology, diluted 1:10) overnight at 4°C. After being washed with phosphate-buffered saline (PBS), the sections were reacted with anti-goat Alexa 488-conjugated antibody (Molecular Probes, # A11078, Lot: 602–1, diluted 1:1,000) and propidium iodide (diluted 1:10,000) for 2 h at room temperature. The cells were observed under a confocal laser scanning microscope (LSM700; Zeiss, Jena, Germany).

In vitro maturation of porcine oocytes

Ovaries were collected at a local slaughterhouse and transported to the laboratory in warm saline (37°C). COCs were aspirated from ovarian follicles of 3- to 5-mm diameter with a 10-ml syringe attached to an 18-gauge needle. Twenty compact COCs with uniform ooplasm were selected in PBS supplemented with 0.1% (w/v) polyvinyl alcohol (PVA). After being washed in PBS-PVA, the 20 COCs were cultured in 200 μl of bovine serum albumin (BSA)-free North Carolina State University 23 (NCSU23) medium (Petters & Wells, 1993) supplemented with 50 μM β-mercaptoethanol, 0.6 mM cysteine (Sigma), 0.5% insulin, 10% (v/v) porcine follicular fluid, pregnant mare serum gonadotropin (10 IU/ml) (Serotropin; Teikokuzouki, Tokyo, Japan), and human chorionic gonadotropin (10 IU/ml) (Puberogen; Sankyo, Tokyo, Japan). The COCs were cultured at 38.5°C in 5% CO_2 in air with maximum humidity. Before analysis of the CNP effect on GVBD occurrence, COCs were pre-cultured in medium with 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP) for 22 h to synchronise nuclear maturation. After the 22-h pre-culture, denuded oocytes (DOs) were prepared from some of the incubated COCs by removing cumulus cells by pipetting. Then, the COC and DO groups were further cultured for 22 h in a medium without hormones and dbcAMP in the presence of various concentrations of CNP (10, 50, 100, 500, or 1000 nM). Each concentration of CNP was prepared as a 100× stock solution in PBS and added into the medium at the indicated final concentration. After 22 h of culture, cumulus cells were removed and oocytes in both groups were fixed in acetic acid/ethanol (1:3) for 48 h. Then, fixed oocytes were stained with 1% aceto-orcein and observed under a phase-contrast microscope to evaluate GVBD induction.

Oocytectomy of porcine COCs

Oocytectomy of oocytes was performed as described by Kimura *et al.* (2002). Briefly, a COC was fixed to the bottom of the dish with precision tweezers, and a puncture was made through the layer of cumulus cells and the oocyte with the tip of a 26-gauge needle. Then, the oocytoplasm was pressed out gradually with the tweezers. Twenty COCs, OXCs, or OXCs with DOs were cultured in 20 μl of NCSU23 medium without dbcAMP and hormones for 24 h.

RNA isolation and cDNA synthesis

After the COCs were cultured for the indicated periods, the oocytes and cumulus cells were separated from the COCs. Cumulus cells were removed from COCs with a glass pipette and transferred into a microtube, washed twice in PBS, and centrifuged at 1,000 rpm for 3 min. Total RNA was extracted from oocytes or cumulus cells with an RNeasy Micro Kit (Qiagen), following the manufacturer's instructions. Reverse transcription was performed in a 20-μl mixture containing 1 μl of cDNA and RNase inhibitor, 4 μl of 5× buffer and dNTP, 2 μl of dithiothreitol (DTT), and 1 μl of reverse transcriptase (SuperScript II; Invitrogen, Carlsbad, CA, USA). The mixture was then incubated at 42°C for 50 min and at 70°C for 15 min.

Reverse transcription-PCR

For detection of *npr2* mRNA in cumulus cells and oocytes, RT-PCR was performed. Specific primers for *npr2* and β-*actin* were designed with the following sequences: *npr2* (NCBI accession no. AY550069), ggcacaggaatcaccttcat (forward) and tgaagcgagtgagatggttg (reverse); β-*actin* (NCBI accession no. NM_001244322), aggtcatcactattggcaac (forward) and actcatcgtactcctgcttg (reverse) (Roh *et al.*, 2007). The generated product size for *npr2* and β-*actin* was 364 bp and 363 bp, respectively. The reaction volume was 20 μl, and *Taq* polymerase (Takara Bio, Otsu, Shiga, Japan) was used. cDNA was amplified using the following program: 35 cycles of denaturation at 95°C, annealing at 57°C for *npr2* or at 57.5°C for β-*actin*, and elongation at 72°C for 45 s).

Real-time PCR quantification

For quantitative analysis of *npr2* mRNA expression, real-time PCR was performed. Total RNA (250 ng) was reverse-transcribed. The specific primers were designed based on the sequences of *npr2* (forward: atggtcaacgccatgccccg, reverse: cccgtgtgcacccctatgcg) and β-*actin* (forward: atcgtgcgggacataag, reverse: ctcgttgccgatggtgat); product sizes of 123 and 251 bp, respectively, were generated. The program consisted of 44 cycles of denaturation at 94°C for 5 s, annealing at 61°C for 20 s, and extension at 72°C for 15 s, with additional extension at 72°C for 5 min. To confirm whether the correct gene was amplified, the obtained products were loaded onto 2% agarose gels and electrophoresed. The *npr2* mRNA expression levels were normalised to the β-*actin* level. The mean sample and endogenous control threshold cycles (Ct) for each sample were calculated with the ($2^{-\Delta\Delta Ct}$) method. The experiments were performed at least three times.

Statistical analysis

Each experiment was replicated three times. Statistical differences between the means of the two groups were analysed using Student's *t*-test. Differences between more than two groups were assessed by analysis of variance followed by a Bonferroni-Dunn test with STATVIEW (Abacus Concepts Inc., Berkeley, CA, USA). *P*-values less than 0.05 were considered to be significant. Data were represented as mean ± standard deviation (SD).

Results and Discussion

As shown in Fig. 1, intense NPR2 immunoreactivity was detected in oocyte surrounded cumulus cells, suggesting the presence of NPR2 in porcine ovarian follicles. We examined the expression of *npr2* mRNA in cumulus cells and oocytes by RT-PCR, and confirmed that *npr2* mRNA is expressed in porcine cumulus cells but not in oocytes (Fig. 2). This result suggests that CNP targets cumulus cells but not the oocytes. In order to demonstrate the hypothesis, oocytes with CNP were cultured *in vitro*. When COCs were cultured with CNP for 9 h after 22 h of pre-culture in medium containing dbcAMP, significant GVBD inhibition was observed at CNP concentrations of more than 10 nM (Fig. 3). In contrast, when DOs were cultured, CNP did not significantly inhibit the GVBD at any concentration. Our result clearly demonstrates that CNP also acts as a meiotic inhibitor in porcine oocytes. Because the bioreactivity of CNP is attributed to a cGMP-dependent cascade (Schulz, 2005), it seems that CNP-generated cGMP in cumulus cells is transported into the oocyte and that it plays an important role in meiotic arrest.

Npr2, the cognate receptor of CNP, is widely expressed, such as in aortic smooth muscle cells (Rahmutula & Gardner, 2005) or the spinal cord (Schmidt *et al.*, 2007). However, there is limited information about the role of CNP in the ovary. Jankowski *et al.* (1997) reported that *cnp* and *npr2* mRNA expression levels in the ovary are modulated by the oestrous cycle in a rat model. In addition, they

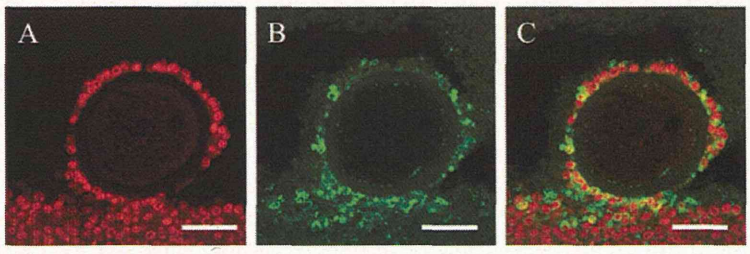

Figure 1 Analysis of natriuretic peptide receptor 2 (NPR2) localisation in porcine ovarian follicles by immunohistochemistry. (*A*) Counterstaining with PI; (*B*) NPR2 staining; (*C*) merged image. Intense positive reaction against anti-NPR2 antibody was observed in oocyte surrounded cumulus cell. Scale bars indicate 100 μm.

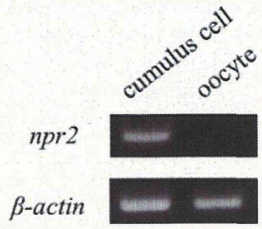

Figure 2 *npr2* mRNA detection in porcine cumulus cells and oocytes. Reverse transcription polymerase chain reaction (RT-PCR) was performed with cDNA prepared from cumulus cells and oocytes. *Npr2* mRNAs were detected only in the cumulus cells but not in the oocytes. The resultant PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

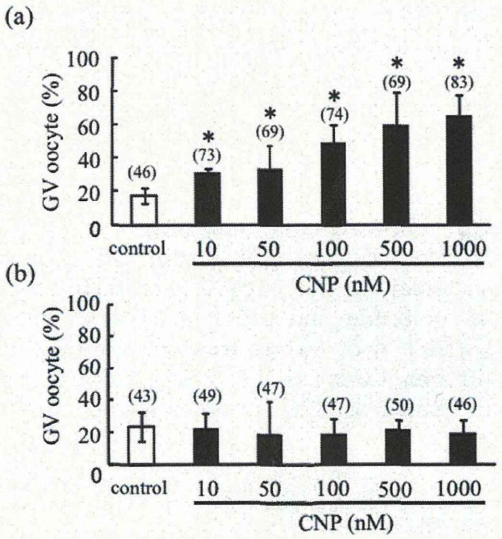

Figure 3 Effect of C-type natriuretic peptide (CNP) on porcine oocyte germinal vesicle breakdown (GVBD) inhibition. Cumulus–oocyte complexes (COCs) (*a*) or denuded oocytes (DOs) (*b*) were further cultured with various concentrations of CNP (10–1,000 nM) for 9 h after 22 h of pre-culture in the presence of dbcAMP. Oocytes were fixed and stained with 1% aceto-orcein, and GVBD occurrence was evaluated. The numbers in parentheses represent the numbers of oocytes examined. Data were analysed by *t*-test. $^*P < 0.05$.

reported changes in *npr2* expression with hormone administration. With oestrogen analogue or equine CG treatment, the *npr2* expression level in granulosa cells was significantly increased. A recent study in mice has shown that CNP stimulates ovarian follicle development (Sato *et al.*, 2012), and a study on *Nppc*- and *Npr2*-mutant mice revealed that lack of the NPPC/NPR2 cascade results in a defective female reproductive system, emphasising the importance of CNP and NPR2 (Kiyosu *et al.*, 2012).

However, while the CNP function has been well established in a rodent model, studies in large domestic animals, including porcine models, have not been done. This result is the first evidence that CNP acts as a meiotic inhibitor in porcine oocytes.

Based on our results showing that *npr2* is expressed in cumulus cells and functions as a CNP receptor, we investigated the regulation mechanism of *npr2* in cumulus cells.

In a previous report in mice, *npr2* expression in cumulus cell was also sustained by the ODPF (Zhang *et al.*, 2010). To demonstrate whether ODPF affects the level of cumulus cell *npr2* expression in the porcine model, we quantitatively compared the level of *npr2* expression in oocytectomised COCs.

As shown in Fig. 4, the relative mRNA expression level of *npr2* was significantly lower than that in the oocytectomised group (OXC) in the COCs group. However, *npr2* mRNA level was restored by co-culture with DOs (OXC + DO), suggesting that porcine ODPF also helps maintain *npr2* expression in cumulus cells. In mice, it has been reported that *npr2* level in cumulus cells was increased by the combination of GDF9, BMP15, and FGF8 (Zhang *et al.*, 2010). In our work, the effects of these factors on *npr2* were not investigated because of the limited availability of these recombinant proteins for porcine studies. Further study is required to identify the main factor(s) involved in the regulation of *npr2* mRNA expression in porcine cumulus cells.

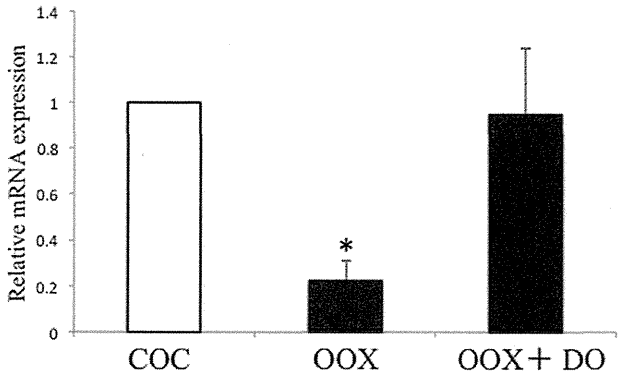

Figure 4 Relative mRNA expression levels of *npr2* in cumulus cells by real-time polymerase chain reaction (PCR) analysis in cumulus–oocyte complexes (COCs), oocytectomised COCs (OXCs), or OXCs with denuded oocytes (DOs) cultured for 24 h. *Npr2* mRNA expression level was normalised to that of β-*actin*. Data are mean ± standard deviation (SD) of three independent experiments. Data were analysed by Bonferroni-Dunn test $^{*}P < 0.05$.

In summary, our data show that CNP acts as a meiotic inhibitor in porcine oocytes through a mechanism indirectly mediated by cumulus cells. Furthermore, OXCs showed decreased levels of *npr2* mRNA expression, suggesting that ODPF also regulates *npr2* expression in the porcine model. Our finding provides a new insight on the underlying mechanism of meiotic resumption in porcine oocytes and raises the possibility of developing techniques to control *in vitro* maturation in porcine oocytes.

Declaration of interest

None.

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ORIGINAL ARTICLE

Distribution of protein disulfide isomerase during maturation of pig oocytes

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ABSTRACT

Oocyte maturation in mammals is characterized by a dramatic reorganization of the endoplasmic reticulum (ER). In mice, the ER forms accumulations in the germinal vesicle (GV) stage and distinctive cortical clusters in metaphase II (MII) of the oocyte. Multiple evidence suggests that this ER distribution is important in preparing the oocyte for Ca^{2+} oscillations, which trigger oocyte activation at fertilization. In this study, we investigated the time course and illustrated the possible functional role of ER distribution during maturation of porcine oocytes by immunostaining with protein disulfide isomerase (PDI). PDI forms clusters in the cytoplasm of oocytes. After immunostaining, PDI clusters were identified throughout the cytoplasm from the GV to metaphase I (MI) stage; however, at the MII stage, the PDI formed large clusters (1–2 μm) in the animal pole around the first polar body. PDI distribution was prevented by bacitracin, a PDI inhibitor. Our experiments indicated that, during porcine oocyte maturation, PDI undergoes a dramatic reorganization. This characteristic distribution is different from that in the mouse oocyte. Moreover, our study suggested that formation of PDI clusters in the animal pole is a specific characteristic of matured porcine oocytes.

Key words: *endoplasmic reticulum, oocyte maturation, porcine, protein disulfide isomerase.*

INTRODUCTION

A hallmark feature of fertilization in mammalian oocytes is the release of Ca^{2+} from intracellular stores. This phenomenon is termed 'Ca^{2+} oscillation', and is essential for preventing polyspermy, initiating egg activation, and recruiting maternal RNAs that initiate protein synthesis after fertilization (Mann *et al.* 2010). Ca^{2+} oscillations originate in the endoplasmic reticulum (ER); therefore, the ER plays an important role during fertilization.

Oocytes need to complete maturation before fertilization. Oocyte maturation is the final stage of oogenesis, during which the fully mature oocyte develops into a fertilization-competent egg. In mammalian oocytes, this process involves the breakdown of the prophase nucleus (germinal vesicle breakdown; GVBD), formation and migration of the metaphase I (MI) spindle to the oocyte cortex, extrusion of the first polar body, and establishment of the metaphase II (MII) spindle. These nuclear changes are accompanied by various cytoplasmic modifications that render the oocyte capable of development after becoming a fertilized egg (FitzHarris *et al.* 2007). The ER exhibits one of these modifications. It undergoes a dramatic reorganization during oocyte maturation in a diverse array of species from marine worms to starfish, *Xenopus*, rodents, bovine and humans (Mann *et al.* 2010). In the GV stage of the mouse oocyte, the ER is continuous with the nuclear envelope and is present throughout the cytoplasm in small accumulations throughout the oocyte interior. During oocyte maturation to the MII stage, the ER changes such that clusters of 1–2 μm in diameter appear in the cortex opposite the meiotic spindle (FitzHarris *et al.* 2007). In the hamster oocyte, between the GV stage and prometaphase I, the number and size of the surface ER masses increased. After prometaphase I, the surface ER masses gradually dispersed into several much smaller ER clusters near the surface (Shiraishi *et al.* 1995). In humans, GV-stage oocytes contain little cortical ER that is not organized into cortical clusters. In contrast, MII oocytes contained large (2–3 μm in diameter), distinct ER clusters throughout the cortex (Mann *et al.* 2010). In porcines, ER formed a thin layer beneath the plasma membrane, and the layer became more evident and wider in the MII-stage oocytes (Maeda & Yagyu 1997); therefore,

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ER clustering is a common feature observed in mature oocytes, but cluster distribution varies among animal species.

ER distribution in cells can be observed using an ER marker. The distribution or expression level of several markers have been identified; the markers include Hsc70, Hsp40, calnexin and protein disulfide isomerase (PDI) (Tatu & Helenius 1995; Zhang *et al.* 1997; Meunier *et al.* 2002), which is a major ER marker. PDI is an oxidoreductase that was identified first as a highly abundant, essential protein in the lumen of ER, where it catalyzes the formation, reduction and isomerization of disulfide bridges in nascent proteins during their folding process (Gilbert 1998). In the present study, to investigate the role of ER during porcine oocyte maturation, we analyzed the distribution and expression level of ER protein PDI.

MATERIALS AND METHODS

In vitro maturation (IVM) of oocytes

Porcine cumulus-oocyte complexes (COCs) were isolated as described by Kawahara *et al.* (2002). COCs with uniform ooplasm and a compact mass of cumulus cells were selected and placed into modified Dulbecco's phosphate buffered saline (PBS) medium containing 5.56 mmol/L glucose (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 0.33 mmol/L sodium pyruvate (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 0.01 mL/mL antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA), and 4 mg/mL fatty-acid-free bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA). This solution was designated as the PB1 medium (Quinn *et al.* 1982). The COCs were washed in the PB1 medium and then cultured in BSA-free North Carolina State University (NCSU)-23 medium for 44 h at 38.5°C in a highly humidified atmosphere of 5% CO_2. For the first 22 h, they were cultured in NCSU-23 medium supplemented with 10 IU/mL pregnant mare serum gonadotropin (PMSG; Serotropin; Aska Pharmaceutical, Ltd, Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (hCG; Puberogen; Daiichi Sankyo, Tokyo, Japan), 0.6 mmol/L cysteine (Sigma-Aldrich, St. Louis, MO, USA), 1.0 mmol/L dibutyryl cyclic adenosine monophosphate (dbcAMP) (Sigma-Aldrich, St. Louis, MO, USA), 5.0 μg/mL insulin (Gibco BRL Life Technologies, Grand Island, NY, USA), 20 mmol β-mercaptoethanol, and 10% porcine follicular fluid. For the subsequent 22 h after the start of culture, COCs were moved to the fresh culture medium, which did not contain dbcAMP and hormone, and cultured for 22 h (total 44 h). During IVM, 25 COCs were cultured with the 250 μL droplets. These culture drops were made in a 35 mm dish (SUMIRON, Osaka, Japan) and were covered with paraffin oil. To inhibit PDI after medium replacement, we added 1.0, 3.0, 5.0, 10 and 20 mmol/L bacitracin (Sigma-Aldrich, St. Louis, MO, USA) to the medium from 22 to 44 h of IVM.

Immunostaining of PDI

GV oocytes were collected immediately after culturing in the second medium, which was considered as 0 h. GVBD, MI and MII oocytes were collected at 6, 12 and 22 h, respectively, after changing to the second IVM medium. The cumulus cells were removed by vortexing the COCs in PB1 medium containing 1 mg/mL hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA). Cumulus-free oocytes were fixed with 4% (w/v) paraformaldehyde for 90 min at 38.5°C. The fixed oocytes were washed three times in PBS and treated overnight with 2% Triton-X100 in PBS at 38.5°C. The oocytes were then briefly washed three times in PBS and treated with the blocking buffer (1% BSA in 0.5% Triton-X100 in PBS) for 60 min at 38.5°C. They were then incubated for 60 min at 38.5°C with rabbit polyclonal antibody to PDI antibody (PDI-ER marker, Abcam, Cambridge, UK) at a dilution of 1:200 in PBS containing 0.1% polyvinyl alcohol (PVA) and 1.0% BSA (PBS-PVA-BSA). After 3 washings with PBS-PVA-BSA, the oocytes were incubated with Alexa Fluor 488-labeled goat anti-rabbit antibody (Molecular Probes, Inc., Eugene, OR, USA) for 60 min at 38.5°C. After repeated washing in PBS, the oocytes were mounted onto a glass slide containing a small volume of PBS-PVA, and stained with 10 μg/mL propidium iodide (PI) in PBS containing 1.0% BSA for a minimum of 60 min to label the chromosomes. Alexa Fluor 488 (488 nm) and PI (615 nm) generate green and red fluorescent signals, respectively. The samples were then viewed using a Bio-Rad MRC-1024 confocal scanning laser microscope mounted on an Axioplan Zeiss microscope. After observing the entire specimen under a microscope, a single photograph was obtained at two or more points. Approximately 100 oocytes were used per treatment. The experiments were repeated a minimum of three times.

Western blot analysis of PDI

For Western blot analysis, PDI, which is a 57-kDa protein, was separated on 8% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted in a semidry blotting apparatus according to the method of Yokoo *et al.* (2002). After electroblotting, the membranes were blocked with 5.0% nonfat milk in Tris-buffered saline Tween-20 (TBS-T) for 60 min at room temperature. After washing with TBS-T, the membranes were incubated with PDI antibody (1:1000) at 4.0°C. The following day, the membranes were washed with TBS-T and reacted with secondary antibody anti-rabbit immunoglobulin G (IgG) (1:20 000; Jackson ImmunoResearch, West Grove, PA, USA) for 60 min at room temperature. The primary and secondary antibodies were diluted in Can Get Signal Immunoreaction Enhancer Solution (Toyobo Co., Ltd, Osaka, Japan). After washing with TBS-T, the chemiluminescence was visualized using an ECL Plus detection system (GE Healthcare (previously Amersham Bioscience), Buckinghamshire, UK). Each treatment was repeated a minimum of three times. Approximately 100 oocytes were used per treatment.

Density of PDI clusters

Density of PDI clusters was measured by the analysis of immunostained images. All images were obtained through confocal microscopy at a point at which the first polar body could be observed. Analyses were performed with ImageJ 1.42 (National Institutes of Health, Bethesda, MD, USA). In order to obtain PDI cluster outlines, we used the 'Threshold' and 'Measure' functions of the software. The densities of the large clusters were calculated as total area of large clusters (1–2 μm)/total area of all PDI clusters.

Statistical analysis

All values were expressed as means with standard errors (SEM) and analyzed using the Tukey-Kramer test following analysis of variance. Differences were considered significant at the 5.0% and 1.0% probability levels. The statistical significance was evaluated using StatView (Abacus Concepts, Inc., Berkeley, CA, USA).

RESULTS

Distribution of PDI during oocyte maturation

To verify the distribution of PDI-labeled ER, we immunostained the ER of oocytes in the GV, GVBD, MI and MII stages. From the GV to the MI stages, PDI was present throughout the cytoplasm (Fig. 1A–C); however, at the anaphase I and telophase I stages, large PDI clusters were present at the animal pole (data not shown), and at the MII stage, the PDI formed distinctively large clusters (approximately 1–2 μm in diameter) in the cytoplasm (Fig. 1D).

Expression of PDI during oocyte maturation

To determine the amount of PDI expression during IVM, we performed Western blot analysis. As shown in Figure 2, PDI was expressed during oocyte maturation. The graph indicates that the amount of PDI expression was not significantly different among the four stages ($P < 0.05$). The graph also indicates that progression of nuclear maturation was associated with an increase in SEM values. The variation of the amount of PDI expression in each stage became larger with the advance of oocyte maturation, and was the largest at MII.

Density of PDI clusters

To verify whether PDI clusters moved with oocyte maturation, we immunostained the ER in oocytes and measured the density of total and large PDI clusters. We observed that large clusters were approximately 1–2 μm in diameter in oocytes (Fig. 3A,B). Moreover, we observed that density of large clusters significantly ($P < 0.05$) increased from immature (GV and MI stages) to matured (MII) stage in oocytes (Fig. 3C).

Determination of the position of PDI clusters

To determine the position of PDI clusters in the oocytes, we next observed MII oocytes using slices from the cortical plain of the animal pole, the equatorial plain, and the cortical plain of the vegetal pole. Distinctive clusters were located in the cytoplasm near the first polar body – the animal pole (Fig. 4A,B). Large clusters were not observed in the vegetal pole (Fig. 4C), but were found in most of the MII-stage oocytes (66.7%, $n = 57$).

Effect of PDI inhibitor on PDI distribution

To verify the importance of PDI activity forming clusters in matured oocytes, we analyzed the effect of different concentrations of the PDI inhibitor, bacitracin, on PDI clusters in MII oocytes. Oocytes exposed to 1 or 3 mmol/L bacitracin (Fig. 5A-b,A-c) formed PDI clusters of similar density to the control (Fig. 5A-a); however, no PDI clusters were observed in oocytes that were exposed to 5 mmol bacitracin (Fig. 5A-d). Moreover, bacitracin significantly inhibited PDI cluster formation in MII oocytes at 5 mmol ($P < 0.05$) (Fig. 5B).

DISCUSSION

In a previous study by Maeda *et al.* changes in the distribution of ER during porcine oocyte maturation have been investigated using fluorescent lipophilic dye (DiI), which labels the continuous ER membrane. They reported that the ER formed a thin layer beneath the plasma membrane. The layer became more evident and wider in the MII-stage oocytes (Maeda & Yagyu 1997). In the present study, we investigated the distribution of PDI, which is an ER marker, during oocyte maturation by immunostaining with PDI antibody, and indicated that PDI formed distinctive large clusters in the cytoplasm. Furthermore, we showed that the change in PDI was not associated with a change of its expression level during oocyte maturation (i.e. only a change in localization takes place).

In the present study, we examined the changes in PDI distribution during porcine oocyte maturation. As shown in Figure 1, the PDI was evenly distributed throughout the cytoplasm from the GV to MI stage of oocyte maturation; however, at the MII stage, the PDI formed distinctively large clusters in the animal pole of the cytoplasm around the first polar body. However, ER protein expression remained at the same level from the GV to MII stage of oocyte maturation (Fig. 2). These results indicate that the distribution of PDI clusters changed during maturation of porcine oocytes which could be related to their cytoplasmic maturation; however, this type of ER distribution seems to be distinct in porcine MII oocytes and different from that in other animal species (Mehlmann *et al.* 1995).

Reports from studies on oocytes of several animal species, from marine worms to starfish, frogs, mice, hamsters and cows, have shown the functional and structural alterations of ER during oocyte maturation (Jaffe & Terasaki 1994; Shiraishi *et al.* 1995; Stricker *et al.* 1998; Terasaki *et al.* 2001; Payne & Schatten 2003; FitzHarris *et al.* 2007; Ajduk *et al.* 2008). In

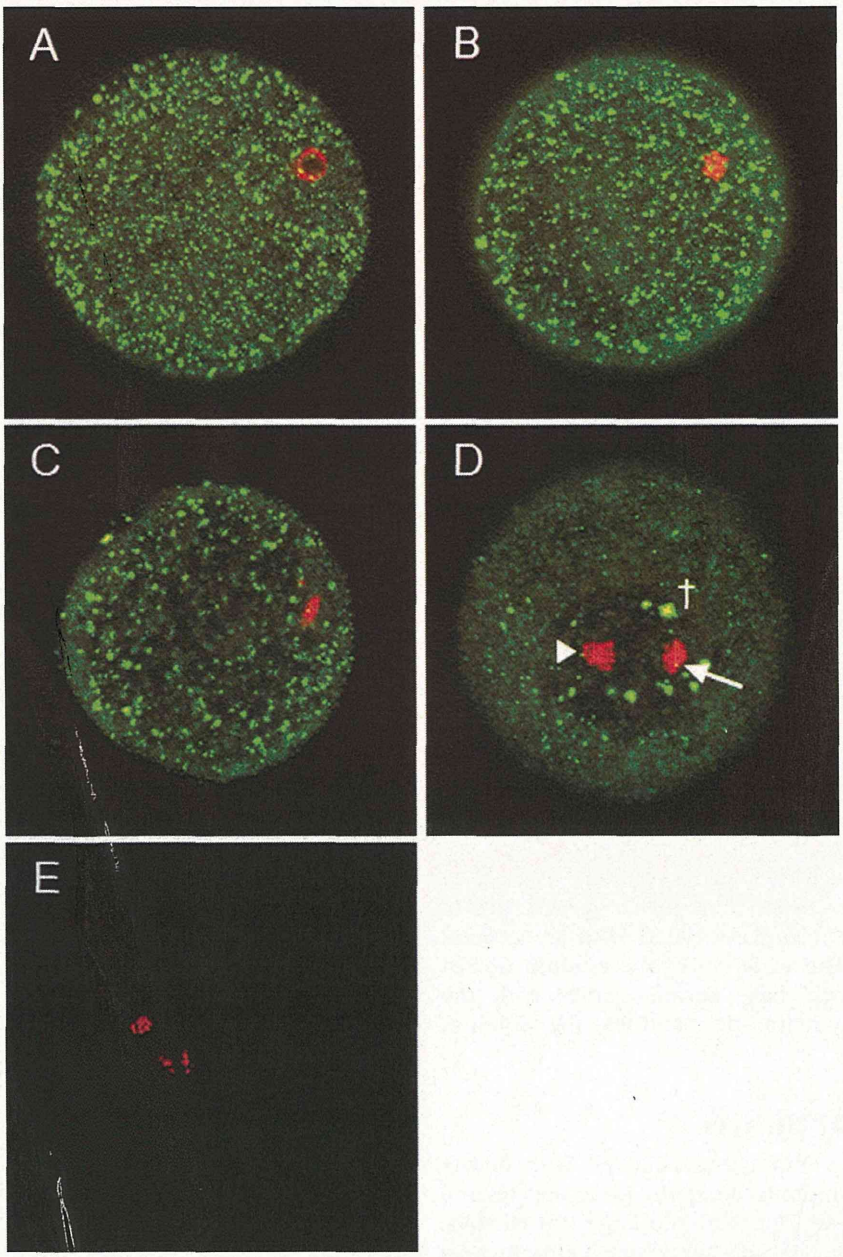

Figure 1 Distribution of protein disulfide isomerase (PDI) during maturation of *in vitro*-matured porcine oocytes. Germinal vesicle (GV) (A) oocyte collected immediately after medium replacement. Germinal vesicle breakdown (GVBD) (B), metaphase I (MI) (C), and metaphase II (MII) (D) collected 6, 12 and 22 h, respectively, after medium replacement. Green and red represent PDI and DNA, respectively. The arrow indicates the first polar body, and the arrowhead indicates the spindle position. The cross indicates the PDI clusters. These images were obtained as whole scans.

GV-stage mouse oocytes, the ER is continuous with the nuclear envelope and is present throughout the cytoplasm, as well as in small accumulations throughout the oocyte interior. During oocyte maturation to the MII stage, the ER changes such that clusters that are 1–2 μm in diameter appear in the cortex opposite the meiotic spindle (vegetal pole) (Mehlmann *et al.* 1995; FitzHarris *et al.* 2007). In the present study, PDI cluster formation was detected in the cortex of the animal pole in porcine MII-stage oocytes (Fig. 4) and this finding was contrary to the distribution of that in mouse oocytes. This fact suggests specific differences in ER distribution in oocytes among mammalian species. The expression of PDI was observed in oocytes without