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PDE5 modulates oocyte spontaneous maturation via cGMP-cAMP but not cGMP-PKG signaling

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1. ABSTRACT

Phosphodiesterase type 5 (PDE5), a cGMP specific, cGMP binding phosphodiesterase, specifically hydrolyzes cGMP to 5'-GMP. Here, we examine the distribution of PDE5 in mouse ovary and its effects on spontaneous maturation of mouse oocytes. PDE5 is present in oocytes and cumulus cells of big, antral follicles. Inhibition of activity of PDE5 significantly and reversibly inhibits spontaneous maturation of cumulus-oocyte complexes (COCs). Suppressive effect of PDE5 on spontaneous maturation of COCs is not blocked by the inhibitor of cGMP-dependent protein kinase (PKG). While Sildenafil, an inhibitor of PDE5 has a poor effect on cGMP levels, it significantly increases cAMP levels. These results suggest that the activity of PDE5 plays a role in regulating spontaneous maturation of mouse oocytes and imply that an interaction between cGMP and cAMP signal is involved in this process.

2. INTRODUCTION

Mammalian oocytes in follicles are arrested at the diplotene stage of the first meiotic prophase, when its nucleus is entitled as germinal vesicle (GV). If an oocyte is removed from an antral follicle and cultured in proper medium *in vitro*, it can spontaneously resume meiosis and progress to second metaphase (1, 2). Spontaneous maturation of oocytes isolated from their follicles can be prevented by including membrane permeable cAMP analogs such as dibutyryl cAMP (dbcAMP) or cAMP phosphodiesterase inhibitors, such as hypoxanthine (HX) or 3-isobutyl-1-methylxanthine (IBMX), in the culture medium (3-6). It is well established that meiotic arrest is regulated by the cAMP levels in the oocyte (5) and that a decrease in oocyte cAMP precedes resumption of meiosis (7, 8).

It is generally accepted that cyclic AMP is a key second messenger in regulating oocyte maturation (7, 9, 10). However, the potential roles of another second messenger cyclic GMP as a cyclic nucleotide in modulating oocyte maturation are less clearly defined. Hubbard and Terranova found that the inhibitory action of cGMP analog 8-Br-cGMP on hamster oocyte maturation is dependent on an intact cumulus, 8-Br-cGMP exhibit no effect on denuded oocytes maturation (11). In starfish, the intracellular cGMP level transiently decreases when oocyte maturation initiates, and exogenously applied cGMP inhibit hormone-induced maturation (12). It has also been reported that the decrease of cGMP levels in rat oocyte is observed during the first hour of spontaneous maturation and that microinjection of cGMP into isolated rat oocyte delays spontaneous maturation (13). Our recent study demonstrates that elevation of cGMP levels by nitroprusside sodium (SNP), a nitric oxide (NO) donor, inhibit mouse oocyte spontaneous maturation (14) and that cGMP-elevating agent atrial natriuretic peptide (ANP) repressed follicle-stimulating hormone (FSH)-induced pig oocyte maturation and cumulus expansion (15). Meanwhile, it is also reported that elevation of cGMP levels by cGMP-elevating agents (8-Br-cGMP, ANP or SNP) can partially overcome the inhibition of FSH on hamster oocyte spontaneous maturation after 2h incubation period (16). The inconsistent effects of cGMP on oocyte spontaneous maturation may result from the difference of concentrations of cGMP-elevating agent used in the experiments and the distinctness of the culture systems.

Intracellular concentration of second messenger cGMP is determined by guanylate cyclases that catalyze the formation of cGMP from GTP and cyclic nucleotide phosphodiesterases (PDEs) which catalyze the breakdown of cGMP. PDE5, which was first purified by Francis and his co-workers from rat lung in 1980 (17), and cloned by McAllister-Lucas *et al.* in 1993 (18), is a cGMP-binding enzyme that specifically hydrolyzes cGMP to 5'-GMP. Inhibition of PDE5 by its inhibitor will lead to accumulation of cGMP. Cyclic GMP has several cGMP targets including cGMP-dependent protein kinases (PKG) and cGMP-regulated PDEs, and cGMP-gated ion channel (19). The most common pathway of cGMP action is mediated by activation of PKG, which phosphorylates specific substrates within cells, influencing their activity and leading to a series of downstream effects.

Cyclic GMP can also affect cell signaling through activation and inactivation of PDEs. It is interesting that cGMP stimulates PDE2 activity (20), whereas inhibits PDE3 activity (21). In some cell systems, PDE3 is inhibited by NO and cGMP, resulting in increase of cAMP concentrations and activation cAMP-dependent signaling (22, 23). It has been reported that PDE3A isoform is a prevalent PDE in mouse oocyte (24) and that its activity in mouse cumulus-oocyte complexes (COCs) increase prior to resumption of meiosis in both spontaneous and gonadotropin-induced maturation (25). It also has been confirmed that oocyte spontaneous maturation is blocked by nonspecific PDE inhibitors and PDE3 inhibitors (26-28). Correspondingly, we put forward the hypothesis that suppression of PDE3A by elevated cGMP levels, as a result of PDE5 inhibition, would lead to inhibition of cAMP degradation and increase of cAMP levels within oocyte, which then facilitates the blockage of meiosis resumption.

PDE5 has been found in mouse several tissues such as lung, cerebellum, heart, brain and kidney (29), but no data have been published for the localization of the enzyme in mouse ovary by far. Moreover, the role of PDE5 on oocyte spontaneous maturation is poorly understood. The aim of the present study is to investigate the expression of PDE5 in mouse ovary and the potential roles of PDE5 in mouse oocyte spontaneous maturation and to explore the mechanisms whereby PDE5 elicits its effects.

3. MATERIALS AND METHODS

3.1. Animal

Prepubertal female mice of the Kun-ming White breed (aging 21-23day, weighing 14-16g) were kept in a room with 14 h/10 h light-dark cycles and fed *ad libitum*. The light starts from 6:00am. The animals were handled by the rules stipulated by the Animal Care and Use Committee of China Agricultural University.

3.2. Reagents

Equine chorionic gonadotropin (eCG) was purchased from Ningbo hormone factory and was dissolved in 0.9% saline solution for administration, M199 was obtained from GIBCO (USA). Hypoxanthine (HX) was purchased from sigma-aldrich USA. Zaprinsat (1,4-Dihydro-5- (2-propoxyphenyl)-7H-1,2,3-triazoli (4,5-d)pyrimidin-7-one; sigma-aldrich USA), the first generation inhibitor of PDE5, was prepared as a stock solution at 36.86mM in dimethylsulfoxide (DMSO) and 10μM was used with oocytes. The second generation PDE5 selective inhibitor sildenafil citrate (1- ((3- (6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo (4,3-d)pyrimidin-5-yl)-4-ethoxyphenyl)sulfonyl)-4-methylpiperazine citrate) was purchased from Scenery Chem. (Hefei, Anhui, China). It was dissolved in DMSO at concentration of 10mM and diluted in culture medium for the experiments. The ultimate concentration of sildenafil used with oocytes was 1μM. The cGMP analogue 8-pCPT-cGMP (8- (4-Chlorophenylthio)-guanosine 3', 5'-cyclic monophosphate sodium salt; sigma-aldrich USA) was directly dissolved in culture medium at 19.62mM and 500μM was used with oocytes. The cell permeable, selective, PKG inhibitor KT5823 was purchased from sigma-aldrich USA, a 2mM stock solution was prepared in ethyl acetate and 1μM was used with oocytes. Another PKG inhibitor Rp-8-pCPT-cGMP (Rp-8-((4-Chlorophenyl)thio)-guanosine-cyclic 3′,5′-hydrogen phosphorothioate triethylammonium salt), obtained from sigma-aldrich USA, was dissolved directly in culture medium and used at concentration of 1μM.

3.3. Isolation and culture of oocytes

The basic medium was M199 culture medium supplemented with 3mg/ml BSA, 0.23mM pyruvic acid sodium, 2mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin. This medium was termed as spontaneous maturation medium (SMM). The medium was

designated as HX medium after the basic was supplemented with 4mM HX. The prepubertal female mice received an intra-peritoneal injection of 5 IU /0.1 ml eCG. Forty-four to 46 hours later, the animals were killed by cervical dislocation. The ovaries were dissected out and placed in HX medium. After the bursa and adhering fat were removed, the ovaries were distributed to dishes containing appropriate HX medium. Oocytes were isolated under a stereomicroscope by manual rupture of big, antral follicles using a pair of 26 gauge needles. The spherical oocytes with intact cumulus cells and germinal vesicle were categorized as cumulus-oocyte complexes (COCs). Denuded oocytes (DOs) were obtained by the repeatedly drawing COCs in and out of a small fine-bore pipette. After washed once in the fresh SMM, oocytes were distributed to 35mm in diameter dish containing 2ml of treatment medium (about 50 oocytes each dish) and were incubated at 37^{o}X in 100% humidity air with 5% CO_2. After *in vitro* culture, COCs were denuded of their cumulus cells by manual pipetting and assessed visually for the resumption of meiosis by using a stereomicroscope. The oocytes with an intact germinal vesicle were classified as GV stage. In contrast, those oocytes without an intact GV were sorted as germinal vesicle breakdown (GVBD) stage. Oocytes with the extruded first polar body were labeled as first polar body (PB1) stage. Oocytes with a dark or fragmented ooplasm or dark cumulus cells were classified as degenerated. Unless otherwise noted, oocytes viability for all groups was maintained more than 95%, which is considered normal. The oocytes maturation rate at each stage was calculated by dividing the number of oocytes at each stage by the total number of oocytes.

3.4. Immunohistochemistry for the expression of PDE5

The mouse ovary was collected and fixed in 4% paraformaldehyde at 4^{o}C. Specimens were subsequently dehydrated with graded alcohols, cleared in xylene and embedded in paraffin. Serial sections were cut in 6μm thickness. Paraffin sections were dewaxed, rehydrated and washed in 0.01 M PBS (pH 7.2-7.4). Antigen retrieval was carried out by high pressure in 0.01% citrate buffer (pH 6.0) for 4×4 min. Sections were first treated with 3% H_2O_2 to quench endogenous peroxidase, blocked with 10% normal goat serum, and incubated with a polyclonal rabbit anti-human PDE5 antibody (1:200 dilution, Cell Signaling Technology, Inc. USA) for overnight at 4 ^{o}C. After thorough rinsing with PBS, sections were incubated with corresponding biotinylated secondary antibody for 1hr at room temperature, and rinsed with PBS, and then incubated in avidin-biotin complex for 1hr. Following several washes in PBS, sections were then developed in diaminobenzidine, and finally washed with distilled water and counterstained with hematoxylin. For control experiments, sections were processed by substituting PDE5 antibody with normal rabbit serum.

3.5. PDE5 detection by western blot analysis

Proteins from lung and ovaries were extracted using M-PER® Mammalian Protein Extraction Reagent (Pierce Biotechnology, Inc. USA) in a Dounce homogenizer. Proteins from COCs, cumulus cells (CCs), and DOs were extracted directly by using 2×SDS sample buffer. All proteins were denatured by heating to 100°C for 5 min. After cooled on ice for 30min and centrifuged at 12,000g for 5 min, total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% stacking gel and a 10% separating gel for 1 h at 160V, and then electrophoretically transferred onto a nitrocellulose membrane for 2 h at 200 mA at 4°C. After blocking for 1 h in TBST buffer (20mM Tris, 137mM NaCl, 0.1% Tween 20, pH7.4) containing 1% non-fat milk, the membrane was incubated overnight at 4°C in TBST containing 1:500 polyclonal rabbit anti-human PDE5 antibody (Cell Signaling Technology, Inc. USA), washed in TBST, and incubated with horseradish-conjugated peroxidase-labeled goat anti-rabbit IgG (1:5000 dilution, Pierce Biotechnology, Inc. USA) for 1 h at room temperature. After the membrane was rinsed thoroughly with TBST, the immunoactive bands were detected using enhanced chemiluminescence according to the manufacturer's specifications (Pierce Biotechnology, Inc. USA). Films were scanned with AlphaImage 2200.

3.6. Cyclic AMP and cGMP accumulation in CCs and DOs

After different incubation time, COCs were mechanically dissociated by manual pipetting with a small fine-bore pipette in M199 medium containing 0.2mM IBMX to obtain CCs and homologous DOs were collected separately. In a volume less than 5μ1, DOs were transferred to 100μ1 of 0.1N HCl. The medium were collected and centrifuged at 8000g for 5min, then the supernatant were pipetted out and 100μ1 of 0.1N HCl was added to CCs. An equal volume of medium was collected and used as a blank. All samples were kept on ice for at least 10min, and then stored at -80 ^{o}C. Prior to RIA, samples were thawed, and centrifuged at 12,000g for 5 min, and the supernatant was collected to a glass tube and dried in an oven. Sample extracts were taken up in 100μ1 50mM sodium acetate (assay buffer, pH 4.75) and assayed for cAMP and cGMP as the kit procedure described. The RIA kit, purchased from Shanghai University of T.C.M, had a sensitivity of 4 fmol of cAMP or cGMP, an intraassay coefficient of variation of less than 8.0%, and an interassay coefficient of variation of less than 15.0%. The blank values were subtracted from all determinations. Measured cAMP and cGMP was proportional to the number of DOs and CCs, which was equal to the number of COCs.

3.7. Statistical analysis

Experiments were performed in triplicate, and each experiment was repeated at least three times. All values were presented as mean ± standard error of the mean (SEM). Meiosis resumption frequencies (%GVBD or %PB1) were subjected to arcsine transformation and analyzed by ANOVA procedure (SAS Institute Inc., Cary, NC, USA) followed by Duncan's multiple range tests. cAMP and cGMP levels were analyzed by means of Student's t test. A confidence level of $P<0.05$ was considered statistically significant

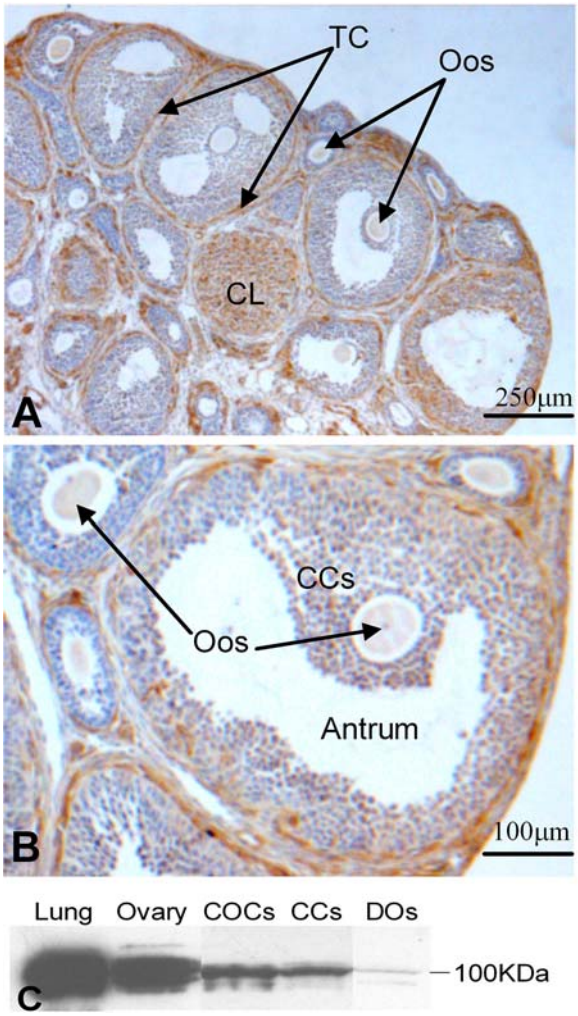

Figure 1. Expression of PDE5 in mouse tissues and cells. A-B, Immunohistochemical analysis. Immunoactivity can be observed in theca cells (TC), corpus luteum (CL), oocytes (Oos) and cumulus cells (CCs) of big, antral follicles. C, Western blot analysis. Lung was used as positive standard. Molecular masses of the revealed bands are about 100KDa. Abbreviations: COCs, cumulus-oocyte complexes; CCs, cumulus cells; DOs, denuded oocytes. Scale bar: A, 250μm; B, 100μm.

4. RESULTS

4.1. Expression of PDE5 in mouse ovary

To study the potential roles of PDE5 in mouse oocyte spontaneous maturation, we first investigated the expression of PDE5 in mouse ovary. Immunohistochemical studies showed that immunoactivity for PDE5 strongly labeled theca cells and corpus luteum. Furthermore, PDE5 positive staining was also present in oocytes of all follicles and cumulus cells of big, antral follicles. No immunostaining of granulosa cells in small follicles was detected (Figure 1A-B). By western blot, we also analyzed the distribution of PDE5 in mouse tissues and cells. As shown in Figure 1C, a major band about 100 kDa was highly expressed, mainly in mouse lung, but also in ovary; it showed a very low level of expression in DOs and an intermediate level in COCs and CCs.

4.2. Effects of PDE5 inhibition on oocyte spontaneous maturation

PDE5 specific inhibitors (sildenafil and zaprinast) were used to assess the effects of PDE5 inhibition on mouse oocyte spontaneous maturation. As shown in Figure 2A, PDE5 inhibition in COCs for 4h significantly ($P<0.05$) decreased the onset of GVBD (58.39±2.51%, 71.80±1.17% and 97.05±0.63% for sildenafil, zaprinast and control respectively, $P<0.05$). After COCs were incubated with PDE5 inhibitors for 24h, both the occurrence of GVBD (zaprinast: 86.54±0.66%, sildenafil: 62.94±2.58%) and the extrusion of PB1 (zaprinast: 28.70±1.79%, sildenafil: 53.13±5.69%) were markedly ($P<0.05$) inhibited compared to the controls (GVBD: 99.06±0.94%, PB1: 79.08±1.68%). To further study the effects of PDE5 inhibitor on COCs spontaneous maturation, we pretreated COCs with PDE5 inhibitor for 4h and then transferred COCs to SMM for another 20h, the result showed that inhibitory actions of PDE5 inhibitor on oocyte spontaneous maturation disappeared (Figure 2B).

4.3. Non-involvement of PKG signal pathway in PDE5 inhibitor modulated oocyte spontaneous maturation

In order to examine the role of cGMP-PKG signal pathway in PDE5 inhibitor-inhibited oocyte spontaneous maturation, we used different PKG inhibitor in the experiment. As shown in Figure 3, Rp-8-pCPT-cGMP, a selective inhibitor of PKG, did not eliminate the inhibitory effects of sildenafil on the occurrence of GVBD and the extrusion of PB1 in COCs at the end of 24h culture. Meanwhile, the suppressive effect of zaprinast was not reversed by another PKG inhibitor KT5823 either. Furthermore, Rp-8-pCPT-cGMP could not overcome the prohibitive action of cGMP analogue 8-pCPT-cGMP on COCs spontaneous maturation. Thus, we concluded that cGMP-PKG signal pathway did not participate in PDE5 inhibitor-modulated mouse oocytes spontaneous maturation.

4.4. Effects of PDE5 inhibitor sildenafil on intracellular cGMP and cAMP levels

To test the hypothesis that cAMP signaling might participate in PDE5 inhibitor mediated oocyte spontaneous maturation, we assessed the effects of PDE5 selective inhibitor sildenafil on accumulation of cGMP and cAMP levels in CCs and DOs, which were isolated from the same COCs. Our results revealed that cGMP levels in DOs and CCs did not significantly increase in response to 1μM sildenafil at any culture time (1h, 2h and 4h), but there was a minor increase of cGMP in DOs at the end of 1h and 2h culture (Figure 4B-D). In contrast, sildenafil (1μM) was found to remarkably promote cAMP levels both in CCs and DOs at the end of culture. cAMP levels in DOs were increased 11-fold (2.6284±0.7582 fmol/DO versus 0.2355±0.0996 fmol/DO, $P<0.05$), 9-fold (1.1463±0.0409 fmol/DO versus 0.1239±0.0522 fmol/DO, $P<0.005$) and 16-fold (1.9744±0.2164 fmol/DO versus 0.1228±0.0251 fmol/DO, $P<0.005$) over control at the end of 1h, 2h, and

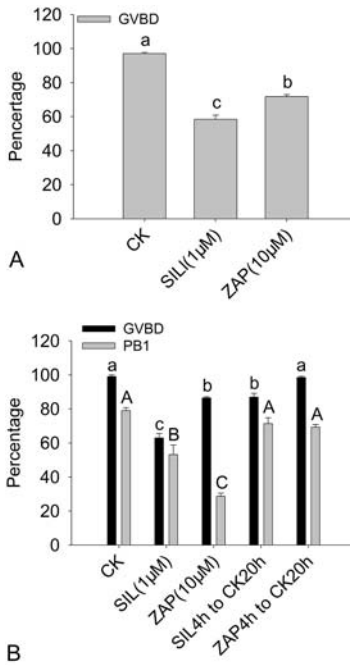

Figure 2. Effects of PDE5 inhibition on oocytes spontaneous maturation. Groups of about 50 oocytes were incubated in the presence or absence of 1μM sildenafil (SIL) or 10μM zaprinast (ZAP) for 4h (A), 24h (B), the percentage of oocytes in stage of GVBD and PB1 were assessed. Bars indicated the percentage of oocytes at GVBD and PB1 stage of meiotic maturation. Values are means ±SEM of three independent experiments. Bars with the same letter are not significantly different according to Duncan's multiple rang test. CK, control group

4h incubation, respectively (Figure 4A). Similarly, sildenafil resulted in an eightfold (5.7103±1.4777 fmol/CC versus 0.6993±0.1253 fmol/CC, $P<0.05$), sevenfold (3.0004±0.1043 fmol/CC versus 0.4015±0.1163 fmol/CC, $P<0.005$) and six fold (3.9755±0.1701 fmol/CC versus

5. DISCUSSION

Our findings demonstrated that PDE5 was present in oocytes and the cumulus cells of big, antral follicles and that the selective inhibition again PDE5 could suppress oocyte spontaneous maturation. The inhibitory effects of PDE5 inhibition were not reversed by the PKG inhibitor (Rp-8-pCPT-cGMP, KT5823). In addition, our study also indicated that PDE5 inhibitor sildenafil significantly increased the levels of intracellular cAMP. These results suggested that not cGMP-PKG signal pathway but an interaction between cGMP and cAMP signaling was involved in PDE5 mediated mouse oocyte spontaneous maturation.

At present, little is known about the expression of PDE5 in mouse reproductive system, especially in mouse ovary. To assess the roles of PDE5 inhibition on oocyte spontaneous maturation, we investigated the expression of PDE5 in mouse ovary. Our immunohistochemical studies and western blot analysis revealed for the first time the existence of PDE5 in mouse oocytes and cumulus cells of big, antral follicles, providing the physiological basis for investigating the effects of PDE5 inhibition on mouse oocyte spontaneous maturation. It should be noted that COCs, CCs and DOs used in immunoblot were isolated from big, antral follicles. The results of western blot analysis were consistent with that of immunohistochemistry.

It was observed that microinjection of cGMP into isolated rat oocyte delayed spontaneous maturation (13). In agreement with this observation, we found in our study that after oocytes were exposed to 8-pCPT-cGMP, a membrane permeable cGMP analogue, for 24h, the percentage of GVBD occurrence and PB1 extrusion were markedly decreased. As COCs expressed a nice bit of PDE5, inhibition of PDE5 by its inhibitor might elicit some effects on COCs spontaneous maturation. Our observations indicated that suppression of PDE5 by sildenafil and zaprinast decreased the occurrence of GVBD of COCs at the end of 4h culture and the percentage of GVBD and PB1 of COCs at the end of 24h incubation. In other words, inhibition of cGMP breakdown by PDE5 inhibitor resulted in the blockage of oocyte spontaneous maturation. The blockage of oocyte spontaneous maturation elicited by PDE5 inhibitor could also be eliminated. After COCs were pretreated with PDE5 inhibitor for 4h and then were transferred to SMM for 20h, the inhibitory actions of PDE5 inhibitor vanished. These results suggested that the suppressive effects of PDE5 inhibitor on COCs spontaneous maturation were reversible.

It is well known that PKG plays a central role in affecting many physiological functions following cGMP elevation. We previously found that the repressive effects of ANP on FSH-induced pig oocyte maturation could be blocked by PKG inhibitor KT5823 (15). It has also been reported that PKG played a vital role in NO donor activated pig oocytes (30). However, in the present study, we found that PKG selective inhibitor KT5823 or Rp-8-pCPT-cGMP did not reverse the inhibitory effect of PDE5 inhibitor on oocyte spontaneous maturation and that the inhibitory effects of cGMP analogue 8-pCPT-cGMP on COCs spontaneous maturation could not be abolished by PKG selective inhibitor Rp-8-pCPT-cGMP. Our results suggested that cGMP-PKG signal pathway was not involved in PDE5-mediated mouse oocyte spontaneous maturation. Our previous studies demonstrated that NO donor SNP could delay the occurrence of GVBD and inhibit the extrusion of PB1 (14). Further investigation indicated that PKG inhibitor KT5823 could not eliminate the actions of SNP either (data not shown). The inconsistent role of PKG signal pathway might be owing to the difference of experimental animal species and culture systems.

As cAMP was the well-characterized second messenger that regulated oocyte maturation (7, 9, 10), we explored the possible interaction between cGMP and cAMP signaling. In our study, after COCs were exposed to

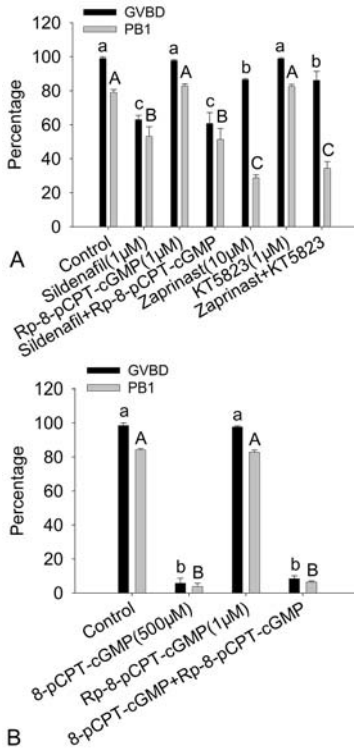

Figure 3. Non-involvement of PKG signal pathway in PDE5 inhibitor modulated COCs spontaneous maturation. At the end of 24h culture the percentage of oocytes in stage of GVBD and PB1 were determined. Bars indicated the percentage of oocytes at GVBD and PB1 stage of meiotic maturation. Values are means ±SEM of three independent experiments. Bars with the same letter are not significantly different according to Duncan's multiple rang test.

PDE5 inhibitor for 4h, the occurrence of GVBD was suppressed (Figure 2A). This suppressive effect of PDE5 inhibitor on GVBD occurrence was similar to that of cAMP-elevating agents such as forskolin (31) or PDE3 inhibitor (32), implying that cAMP signaling might be involved in PDE5-mediated oocyte spontaneous maturation.

In order to get further evidences on the interaction between cGMP and cAMP signaling, we assessed the effects of sildenafil, a very potent inhibitor of PDE5, on the accumulation of cGMP and cAMP levels in CCs and DOs. Our findings revealed that there was a minor but not significant effect of sildenafil on cGMP levels in CCs and DOs at the end of culture (1h, 2h). In contrast, sildenafil was found to increase cAMP levels significantly ($P<0.05$) in both CCs and DOs at the end of culture (1h, 2h and 4h). These data provided the firsthand proof for the stimulatory effects of PDE5 inhibition on cAMP levels, highlighting the fact that an interaction between cGMP and cAMP signaling might exist in PDE5 modulated mouse oocyte spontaneous maturation. The effects of sildenafil on accumulation of cGMP and cAMP levels in CCs and DOs were reported for the first time, although the similar results in other tissues such as isolated human corpus cavernous smooth muscle (33) had been found previously. The poor effect of sildenafil on cGMP accumulation in CCs and DOs was in accordance with the results of Jeremy who reported that sildenafil had little effect on cGMP in rabbit corpus cavernous in the absence of NO donor (34).

Elevation of cAMP levels in DOs by PDE5 inhibitor sildenafil raised the hypothesis that the effects of PDE5 inhibition on oocyte spontaneous maturation might partly or indirectly include actions of the cAMP second messenger system. Previous study indicated that the PDE expressed in the mouse oocyte was PDE3A and that activity of this enzyme was involved in the control of resumption of meiosis (24). Our study revealed the expression of PDE5 in the mouse oocyte. The exact mechanism by which interaction between cGMP and cAMP signaling occurs was not clear, but the coexpression of PDE3 and PDE5 in oocyte might lead to the potential to target PDE3 activity by PDE5 inhibition. Our results did present here that an increase of cGMP level was observed in DOs after COCs were incubated with sildenafil for 1h and 2h. Thus, an increase in cGMP by inhibition of PDE5 was likely to elicit an inhibitory effect on PDE3, resulting in an elevation of intracellular cAMP and, finally, a blockage of oocyte meiotic maturation. Comprehensive studies showed that inhibitors of cGMP-inhibited PDE3, such as milrinone, and cilostamide, had an inhibitory effect on oocyte spontaneous maturation (26, 27, 32, 35). Moreover, it has been reported that PDE3A activity increased prior to resumption of meiosis during spontaneous maturation of rat COCs (25) and that oocytes of PDE3A deficient mice failed to undergo spontaneous maturation *in vitro* (36). Taken together, results of the present study and other literature data contributed evidences that the crosstalk between cGMP and cAMP signaling might be involved in the regulation of PDE5 modulated mouse oocyte spontaneous maturation.

Although it was not clear how sildenafil increased cAMP levels in isolated CCs, elevation of cAMP levels in CCs might be responsible for spontaneous maturation of COCs. It has been reported that oocyte maturation involved compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells (26). In the present study, when COCs were pretreated with PDE5 inhibitors for 4h and then transferred to SMM for another 20h, the inhibitory effects of these drugs on COCs spontaneous maturation were almost complete reversed. This reversion may be due to the initial rise in cAMP levels in CCs followed by a decline in DOs cAMP levels by local PDEs. Conversely, when COCs were incubated with sildenafil for 24h all along, cAMP levels of CCs and DOs were both promoted all through. The promotive effect of increased cAMP levels in CCs might be covered by the suppressive effect of elevated cAMP levels in DOs, leading to inhibition of COCs spontaneous maturation.

In conclusion, our present results demonstrated that PDE5 was expressed by oocytes and cumulus cells of big, antral follicles and that selective inhibition of PDE5

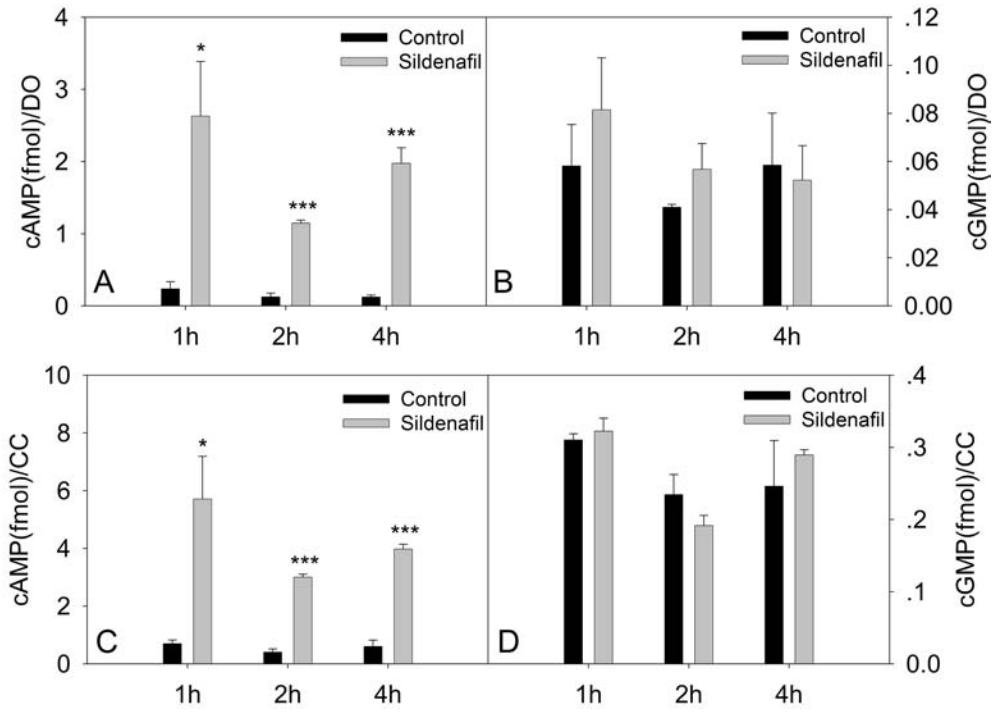

Figure 4. Effect of PDE5 inhibitor sildenafil (1μM) on accumulation of cAMP (A, DOs; C, CCs) and cGMP (B, DOs; D, CCs) levels. The data represent means ±SEM of three independent experiments. *$P<0.05$, ***$P<0.005$ according to *t* test.

could inhibit mouse oocyte spontaneous maturation in a PKG-independent manner and suggested that the inhibitory effects of PDE5 inhibition were elicited via cGMP-cAMP signaling.

6. ACKNOWLEDGEMENTS

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Abbreviations: PDE5: phosphodiesterase type 5, COCs: cumulus-oocyte complexes, PKG: cGMP-dependent protein kinase, GV: germinal vesicle, HX: hypoxanthine, IBMX: 3-isobutyl-1-methylxanthine, SNP: nitroprusside sodium, NO: nitric oxide ANP: atrial natriuretic peptide, DMSO: dimethylsulfoxide, SMM: spontaneous maturation medium, DOs: Denuded oocytes, CCs: cumulus cells, GVBD: germinal vesicle breakdown, PB1: first polar body,

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