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FSH and LH induce progesterone production and progesterone receptor synthesis in cumulus cells: a requirement for meiotic resumption in porcine oocytes

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The aim of this study was to investigate the role of progesterone in the meiotic resumption of porcine oocytes. Progesterone production and progesterone receptor (PR) immunoreactivity in cumulus cells were not detected in porcine cumulus–oocyte complexes (COC) when observations were made either just after collection from the follicles or after 28 h cultivation without LH and FSH. However, the addition of LH and FSH induced PR expression in cumulus cells, concomitant with increased progesterone production. To assess the role of progesterone in the COC, an inhibitor of progesterone production, aminoglutethimide (AGT), was administered. The addition of AGT to the medium with LH and FSH significantly suppressed progesterone production in a dose-dependent fashion. When COC were cultured with LH, FSH and 0.5×10^{-3} mol/l AGT, almost complete inhibition of progesterone production and of germinal vesicle breakdown (GVBD) was seen. However, this inhibitory effect on GVBD was overcome by additional progesterone. Moreover, 0.5×10^{-3} mol/l AGT also suppressed the reduction in connexin43, a gap junctional protein, in cumulus cells after 28 h cultivation, and increased the level of cyclic AMP in oocytes. These results support the hypothesis that the binding of progesterone, which was secreted by LH- and FSH-stimulated cumulus cells, to its newly synthesized receptor induces GVBD in porcine oocytes, possibly through a reduction of connexin43 in cumulus cells.

Key words: connexin43/GVBD/progesterone/progesterone receptor/porcine oocytes

Introduction

During in-vitro meiotic maturation of cumulus–oocyte complexes (COC), progesterone is produced by the cumulus cells, and the level of progesterone is increased by stimulation with LH, FSH or forskolin in humans (Chian *et al*., 1999) and pigs (Racowsky, 1985). It has been reported that a high concentration of progesterone in follicle fluid induces meiotic maturation of rhesus monkey oocytes (Morgan *et al*., 1990). In porcine and bovine oocytes, the addition of progesterone into a maturation medium stimulates meiotic re-initiation, regardless of the presence or absence of gonadal hormones (Sirotkin, 1992; Eroglu, 1993). However, other reports have shown no statistically significant stimulation of meiotic resumption by progesterone in the case of porcine oocytes (McGaughey, 1977). Moreover, progesterone has been reported to suppress spontaneous meiotic maturation in mouse oocytes (Barrett and Powers, 1993). Thus, it is unclear whether or not progesterone is required for in-vitro meiotic resumption in mammalian oocytes.

Many of the biological activities of progesterone are mediated by an intracellular receptor, a hormonally regulated DNA-binding protein that belongs to a superfamily of ligand-activated transcription factors (Beato *et al*., 1989; O'Malley, 1990). The expression of progesterone receptor (PR) mRNA can be induced by either LH or FSH through a cAMP-mediated pathway in rat pre-ovulatory follicles (Natraj and Richards, 1993; Park-Sarge and Mayo, 1994). In the human ovary, PR mRNA expression in antral follicles is significantly higher than that in pre-antral follicles (Revelli *et al*., 1996). Recently, it was reported (Slomczynska *et al*., 2000) that PR are not detected in porcine granulosa cells of small antral follicles, however, treatment of the small follicles with LH or FSH can induce the expression of the PR protein. Although it is known that in-vivo expression of PR protein in follicle cells is regulated by both LH and FSH, there is no description of the expression of the PR protein in cumulus cells surrounding oocytes during in-vitro meiotic maturation of COC in primates and pig.

Some of the meiosis inhibitory factors transported into oocytes via numerous gap junctions are synthesized by the cumulus and granulosa cells that are attached to oocytes (Downs and Eppig, 1984; Downs *et al*., 1986; Isobe *et al*., 1996). A loss of the cumulus-to-cumulus cell gap junction preceding the induction of meiotic resumption has been reported in rat COC (Larsen *et al*., 1986, 1987). In pigs, a significantly positive correlation has been observed between the proportion of oocytes undergoing germinal vesicle breakdown (GVBD) and that of COC exhibiting a loss of the gap junctional communication between the cumulus cells of outer layers (Isobe *et al*., 1998; Isobe and Terada, 2001). In our previous study (Shimada *et al*., 2001), a disruption of gap junctional communication in the outer layers of cumulus cells resulted in the depletion of connexin43 expression in those layers. Furthermore, connexin43 expression in the rat endometrium is regulated by progesterone during early pregnancy (Grummer *et al*., 1994). It has also been shown that progesterone down-regulates the expression of the connexin43 gene in human myometrial cells (Zhao *et al*., 1996). These studies support the idea that progesterone induces a reduction in connexin43

expression via the PR-mediated transcription pathway in the outer layers of cumulus cells, resulting in meiotic resumption in porcine oocytes.

In order to investigate the role of progesterone and the PR in cumulus cells during meiotic resumption in porcine oocytes, the present study examined the effects of gonadotrophins and/or aminoglutethimide (AGT) on the production of progesterone, the PR, and connexin43 in cumulus cells, as well as their effects on cAMP levels and the induction of meiotic resumption in oocytes.

Materials and methods

Isolation and culture of porcine COC

Porcine ovaries were collected from 5–7 month old pre-pubertal gilts at a local slaughterhouse and were transported within 1.5 h to the laboratory in 0.85% (w/v) NaCl containing 0.1 mg/ml kanamycin (Meiji Seika, Tokyo, Japan) at ~30°C. The surfaces of intact healthy antral follicles measuring 3–8 mm in diameter were cut with a razor blade and oocytes were collected with a surgical blade used to scrape the inner surface of the follicle walls. The oocytes collected were placed in pre-warmed phosphate-buffered saline (PBS) (pH 7.4) supplemented with 0.1% (w/v) polyvinylpyrrolidone (Sigma Chemical Co., St Louis, MO, USA). Oocytes with evenly granulated cytoplasm and at least four layers of unexpanded cumulus oophorus cells (COC) were selected under a stereomicroscope and were washed three times with maturation medium. Twenty COC were cultured for 28 h in 500 µl of maturation medium, i.e. basic medium supplemented with 0.6 µg/ml porcine FSH (Sigma) and 1.3 µg/ml equine LH (Sigma), at 39°C in a humidified atmosphere of 5% CO_2 in air. The basic medium was modified NCSU37 (Petters and Reed, 1991) containing 10% (v/v) fetal calf serum (Gibco BRL, Grand Island, NY, USA), 7 mmol/l taurine (Sigma), 2% (v/v) essential amino acids (Gibco), and 1% (v/v) non-essential amino acids (Gibco).

Treatment of COC with progesterone

Some COC were cultured for 28 h in the basic medium supplemented with 0, 10, 100 and 1000 ng/ml progesterone (Sigma). Other COC were cultured for 28 h in the maturation medium supplemented with 0, 10, 100 and 1000 ng/ml progesterone. Progesterone was dissolved in ethanol (Katayamakagaku, Osaka, Japan) at 1 mg/ml, and stored at 4°C. Each final concentration was obtained by dilution in either the basic medium or the maturation medium.

Treatment of COC with aminoglutethimide

COC were cultured for 28 h in maturation medium supplemented with 0, 0.2, 0.5 or 1×10^{-3} mol/l AGT. Since AGT also suppressed the production of estradiol-17β (E_2) in porcine COC (data not shown), some COC were cultured with both 0.5×10^{-3} mol/l AGT and E_2 (Sigma) for 28 h. In order to evaluate the reversible effects of progesterone on the inhibitory effects of AGT, COC were cultured with both 0.5×10^{-3} mol/l AGT and progesterone for 28 h. AGT was dissolved in DMSO (Sigma) at 1 mol/l, and was stored at –20°C. Each final concentration was obtained by dilution in the maturation medium. As a control, inhibitor-free medium was prepared by adding 0.098% (v/v) DMSO to the basic maturation medium. This concentration of DMSO had no adverse effect on porcine oocyte maturation (Shimada *et al*., 1998). E_2 was dissolved in ethanol at 0.1 mg/ml, and was stored at 4°C. Each final concentration (10 and 100 ng/ml) was obtained by dilution in the maturation medium.

Assessment of nuclear maturation

After cultivation of COC, the oocytes were freed from the cumulus cells, and mounted on slides. Oocytes were fixed with acetic acid/ethanol (1:3, v/v) for 48 h, and stained with aceto-lacmoid before examination under a phase-contrast microscope (×400) for the evaluation of their chromatin configuration.

Quantification of progesterone in medium by high-performance liquid chromatography (HPLC)-UV analysis

Quantification of progesterone by HPLC-UV was based on published procedures (Purdy *et al*., 1980). The medium in which COC were cultured for 28 h was collected into plastic tubes and centrifuged at 10 000 *g* for 20 min. The resulting supernatant was stored at –80°C until use. Before analysis, 0.5 ml of the medium sample was saponified by the addition of 0.3 ml of 0.3 mol/l NaOH (Nakarai tesque, Kyoto, Japan) and was left to stand for 10 min. The progesterone was then extracted from the saponified sample by mixing for 5 min with 10 ml dichloromethane (Nakarai). After centrifugation at 3000 *g* for 15 min at 5°C, the 10 ml of dichloromethane fraction was collected into a disposal tube and the solvent from this fraction was removed by vacuum extraction for 120 min at 5°C using a centrifugal concentrator (VC-96N, TAITEC, Tokyo, Japan). Samples were reconstituted in 100 µl of 50% (v/v) methanol solution.

The samples were separated using a reverse-phase Capcell Pak column (2.0×100 mm) (Shiseido, Tokyo, Japan). Column temperature was kept at 40°C by a temperature controller (TSK CO-8000; Tosoh, Tokyo, Japan). The solvent delivery system (TSK CCPD; Tosoh) contained 50% (v/v) methanol solution. The solution was filtered by 0.45 µm Millicup-HV filter (Millipore, Mississauga, Canada) and was degassed. The flow rate was adjusted to 200 µl/min. Samples (100 µl) were injected onto columns using an auto sampler (AS-8020; Tosoh). The detection was performed at 240 nm using a UV detector (UV 8020; Tosoh) and peak heights were measured using a computer integrator (Sic chromatocorder 11; Tosoh).

A standard solution of progesterone (1 µg/ml) (Sigma) was prepared in 50% (v/v) methanol solution and was kept at 4°C. Samples were diluted in 50% (v/v) methanol solution to various concentrations prior to analysis.

Immunofluorescent analysis of PR

COC were fixed with 4% (w/v) paraformaldehyde–PBS (pH 7.4) at room temperature for 30 min, rinsed three times with PBS, and then permeabilized with 0.5% (v/v) Triton X-100-PBS for 30 min. COC were blocked by 5% (w/v) bovine serum albumin (Sigma) in 0.1% (v/v) Tween 20-PBS (T-PBS) and were then incubated overnight at 4°C with anti-human PR monoclonal antibody (which binds to both the A and B forms of PR, clone 1A6; Novocastra Lab., Newcastle upon Tyne, UK) (1:50) in T-PBS containing 5% (w/v) BSA. Samples were rinsed with T-PBS and incubated with a fluorescein-conjugated goat anti-mouse IgG antibody (Sigma) diluted 1:50 in T-PBS containing 5% (w/v) BSA for 2 h at 38°C. The COC were mounted on a slide with 10 µl of antifade solution (SlowFade Antifade Kit; Molecular Probes, Oregon, USA) to preserve the fluorescence and then the COC were observed by fluorescent microscopy (Olympus; IMT-2, Tokyo, Japan). At least 30 COC incubated with anti-PR antibody were observed.

Detection of connexin43 by immunoblotting analysis

Immunoblot detection of connexin43 was performed as described previously (Shimada and Terada, 2001). After being cultured for 28 h, COC were separated into groups of either oocytes or cumulus cells. 1×10^4 cumulus cells isolated from the COC were pelleted by centrifugation (700 *g*, 15 min, 4°C) and the supernatant was discarded. The cumulus cell pellets were resuspended in 5 µl of cell lysis buffer [20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% (v/v) Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 1 µg/ml leupeptin, and 1 mmol/l phenylmethylsulphonyl fluoride (PMSF; Sigma)]. Except for PMSF, the drugs were purchased from New England Biolabs (Beverly, MA, USA). The samples were frozen in liquid nitrogen and then sonicated using an ultrasonic disruptor (UD-200; Tomy, Tokyo, Japan) fitted with a Cup Horn (CH-0633; Tomy); three cycles were performed for 25 s each at 1°C. Cell extracts were frozen and stored at –80°C until use.

The cumulus cell extract was diluted 2-fold with 2×Laemmli sample buffer (Laemmli, 1970). After denaturing by boiling for 5 min, 5 µl of each protein sample from 5×10^3 cumulus cells was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel (Pharmacia Biotech, Uppsala, Sweden). Samples were then transferred onto a PVDF membrane (Amersham, Arlington Heights, IL, USA) using the PhastTransfer system (Pharmacia Biotech). The membrane was blocked using SuperBlock blocking buffer (Pierce, Rockford, IL, USA), then incubated with mouse monoclonal anti-connexin43 antibody (Chemicon International, Temecula, CA, USA) at 1:2000 dilution overnight at 4°C in 10% (v/v) SuperBlock blocking buffer in T-PBS. After three washes in T-PBS, the membrane was treated with horse-radish peroxidase-labelled anti-mouse IgG (1:7000, Amersham) in 10% (v/v) SuperBlock blocking buffer in T-PBS for 1 h at

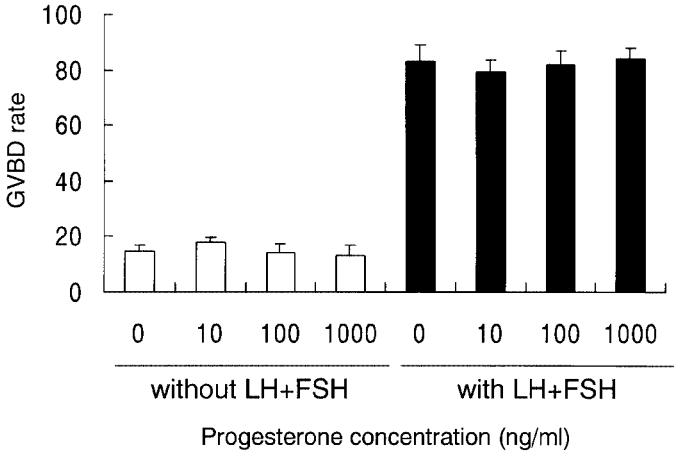

Figure 1. Effects of progesterone on germinal vesicle breakdown (GVBD) in porcine oocytes cultured for 28 h in a basic medium with or without LH + FSH.

room temperature. After three washes of 10 min each with T-PBS, peroxidase activity was visualized using the ECL Plus Western blotting detection system (Amersham), according to the manufacturer's instructions. The intensity of the bands was analysed using a Gel-Pro Analyzer (Media Cybernetics, MD, USA). Each independent experiment was repeated three times.

Quantification of cAMP by HPLC-UV analysis

Oocytes were stripped of cumulus cells by repeatedly aspirating COC through a glass pipette in the presence of 50 µmol/l IBMX (Sigma) in the basic medium. Forty denuded oocytes were transferred to a 100 µl assay buffer (0.01 mol/l ammonium acetate, pH 6.7) containing 50 µmol/l IBMX. The samples were stored at –80°C until assayed.

Oocyte extracts were separated using a reverse-phase Eicompak CA-5DS column (2.1×150 mm) (Eicom, Kyoto, Japan). The column temperature was kept at 25°C by a temperature controller (TSK CO-8000; Tosoh). The solvent delivery system contained 97.2% (v/v) 0.01 mol/l ammonium acetate (Nakarai) and 2.8% (v/v) acetonitrile (Nakarai/Nakalai tesque), pH 6.7. The buffer was filtered with a 0.45 µm Millicup-HV filter and was degassed. The flow rate was adjusted to 200 µl/min. Samples (100 µl) were injected onto the column using an autosampler. The detection was performed at 254 nm using a UV detector; peak heights were measured using a computer integrator.

A standard solution of 100 µmol/l cAMP (Sigma) was prepared in the assay buffer and kept frozen at –80°C. Samples were diluted in the assay buffer prior to analysis.

Statistical analysis

Statistical analyses of all data, including three or four replicates for comparison, were carried out using one-way analysis of variance followed by a least significant difference test using Statview (Abacus Concepts, Inc., Berkeley, CA, USA). All percentage data were subjected to arcsine transformations before the statistical analysis was performed.

Results

Effect of progesterone on GVBD in porcine oocytes

To investigate the effects of progesterone on GVBD in porcine oocytes cultured with or without LH + FSH, COC were cultured for 28 h in a medium supplemented with 0, 10, 100 or 1000 ng/ml progesterone. The percentage of oocytes undergoing GVBD was low during culture without LH + FSH, and the addition of progesterone had no effect. In the presence of LH + FSH, oocytes exhibited a high rate of GVBD, but again the addition of progesterone had no effect (Figure 1). Thus, the addition of progesterone alone into medium without LH + FSH did not accelerate GVBD, and its addition did not affect GVBD which was stimulated by LH + FSH in porcine oocytes.

Table I. Effects of gonadotrophins on the level of progesterone secreted into medium by cumulus–oocyte complexes (COC) and intensity of progesterone receptor (PR) signal in COC

Culture time	Medium	Progesterone concentration (ng/ml)	COC with high PR immunoreactivity* (%)
0 h		ND†	10[c]
28 h	Basic medium‡	7.8 ± 1.5[a]	14[c]
	Maturation medium§ (containing LH + FSH)	89.6 ± 5.8[b]	88[d]
	Medium with AGT¶ (containing LH + FSH and AGT)	9.5 ± 2.6[a]	76[d]

*Numerous intense signals for PR immunoreactivity were present in all layers of cumulus cells (Figure 3d').
†Not detected by this method.
‡The basic medium in which COC were cultured for 28 h.
§The maturation medium in which COC were cultured for 28 h.
¶The medium in which COC were cultured with LH + FSH and 0.5×10^{3} mol/l aminoglutethimide for 28 h.
[a,b; c,d]Rows with no common superscript are significantly different ($P < 0.05$).
AGT = aminoglutethimide.

Progesterone production and PR expression in cumulus cells surrounding oocytes

The concentration of progesterone in medium in which COC had not been cultured could not be detected using this method. The level of progesterone in the medium in which COC had been cultured for 28 h without LH and FSH was 7.8 ± 1.5 ng/ml. However, this concentration significantly increased to 89.6 ± 5.8 ng/ml in medium after cultivation of COC with LH + FSH for 28 h (Table I).

Immunofluorescence studies showed very faint signal intensities in control COC exposed to only fluorescein-conjugated goat anti-mouse IgG antibody (Figure 2a'). Weak immunoreactivity for PR was recognizable in COC just after collection, in all layers of the surrounding cumulus cells (Figure 2b'). COC cultured with LH + FSH for 28 h exhibited numerous and intense PR signals in all the layers of cumulus cells (Figure 2d'); however, in COC cultured for 28 h without LH + FSH, the PR immunoreactivity signal was weak in both cumulus cells and oocytes (Figure 2c'). Furthermore, COC were classified according to the location and number of positive signals for PR: Category 1, a weak fluorescence was detected in the COC (Figure 2b', c'); and Category 2, numerous and intense PR signals were clearly visible in all the layers of cumulus cells (Figure 2d'). The proportion of COC in Category 2 was very low just after collection from their follicles and after 28 h cultivation without LH + FSH (Table I). However, 28 h cultivation with LH + FSH led to an increase in the proportion of COC in Category 2 (Table I). Additionally, 0.5×10^{-3} mol/l AGT did not decrease the high proportion of Category 2 COC seen after 28 h cultivation with LH + FSH (Table I).

The effects of AGT on progesterone production and GVBD in COC cultured with LH + FSH

The addition of AGT to the medium with LH + FSH significantly suppressed the production of progesterone in a dose-dependent fashion during 28 h cultivation of COC (Figure 3).

As shown in Figure 4, the proportion of oocytes exhibiting GVBD significantly decreased as the concentration of AGT increased in medium with LH + FSH. The inhibitory effects reached a plateau and no significant difference was found between 0.5 and 1×10^{-3} mol/l AGT treatments (Figure 4). When COC

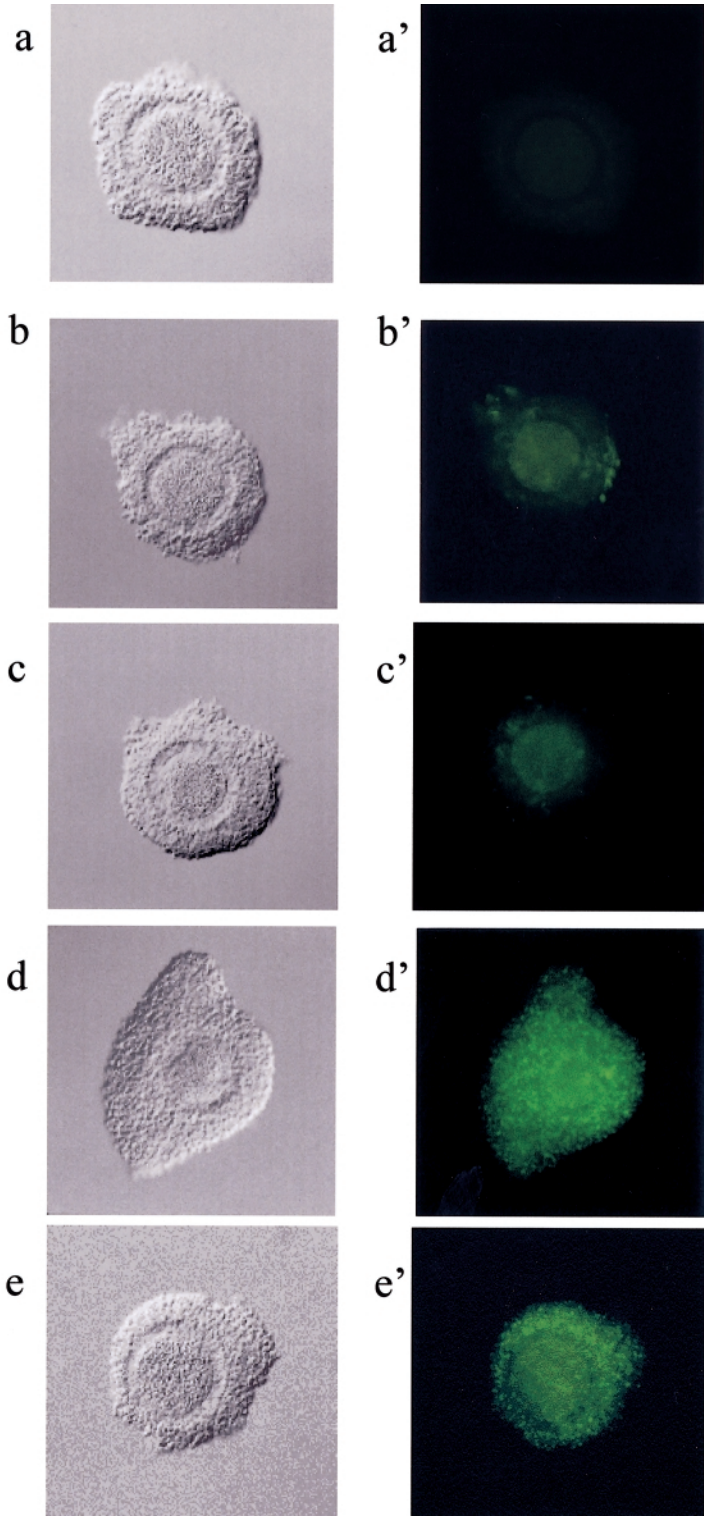

Figure 2. Location of progesterone receptor (PR) in porcine cumulus–oocyte complexes (COC) detected by indirect immunofluorescence. (**a**–**e**) Image obtained by differential interference microscopy. (**a'**–**e'**) Image obtained by indirect immunofluorescence of anti-PR antibody. (**a**, **a'**) Control samples in which no primary antibody was used. (**b**, **b'**) COC immediately recovered from the follicles. (**c**, **c'**) COC cultured for 24 h in basic medium. (**d**, **d'**) COC cultured for 24 h in basic medium supplemented with LH + FSH. (**e**, **e'**) COC cultured for 24 h in basic medium supplemented with LH + FSH + 0.5×10^{-3} mol/l aminoglutethimide. Scale bar = 100 μm.

were cultured in the LH + FSH-containing medium supplemented with 0.5×10^{-3} mol/l AGT and E_2, the E_2 could not overcome the inhibitory effects of AGT on oocytes exhibiting GVBD (Figure 4). In contrast, the addition of 100 and 1000 ng/ml progesterone into the LH + FSH-containing medium supplemented with 0.5×10^{-3} mol/l AGT resulted in an increased proportion of oocytes exhibiting GVBD (Figure 4). Oocytes cultured in the LH + FSH-containing medium with both AGT and progesterone resumed meiosis in similar proportion to that of oocytes cultured in the medium lacking both AGT and progesterone (Figure 4). This result

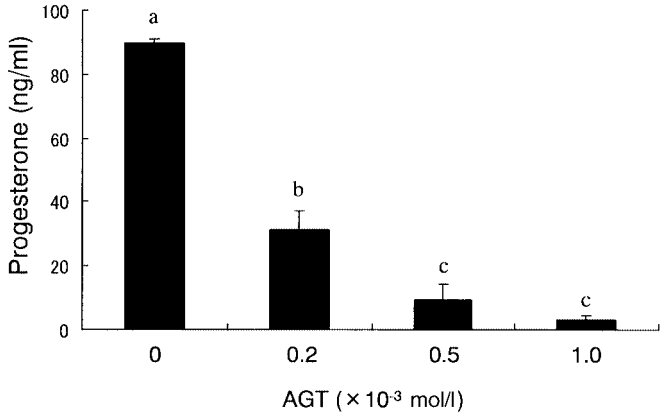

Figure 3. Effects of addition of aminoglutethimide (AGT) into the medium with LH + FSH on progesterone production in porcine cumulus–oocyte complexes (COC) cultured for 28 h. [a–c]Columns with no common superscripts were significantly different ($P < 0.05$).

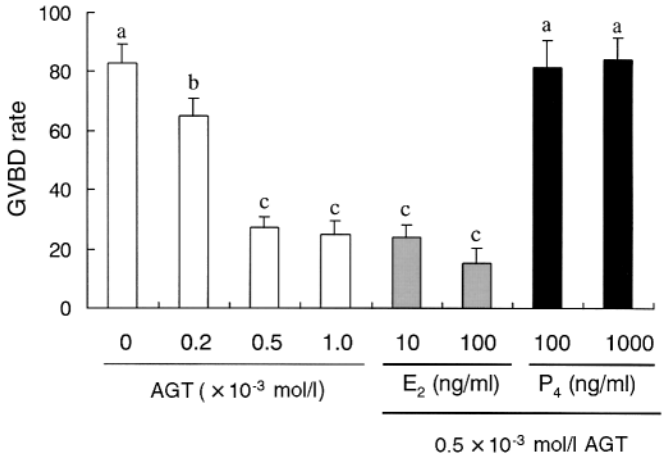

Figure 4. Effects of addition of aminoglutethimide (AGT) with or without steroids on gonadotrophin-stimulated germinal vesicle breakdown (GVBD) in porcine oocytes cultured for 28 h. [a–c]Columns with no common superscripts were significantly different ($P < 0.05$). E_2 = estradiol-17β; P_4 = progesterone.

indicates that the inhibitory effect of AGT on GVBD was fully overridden by the addition of progesterone.

Changes in connexin43 in cumulus cells and cAMP level in oocytes after culture of COC in medium with LH + FSH and AGT

Immunoblotting analysis of extracts of cumulus cells separated from COC just after collection from their follicles revealed that three bands of connexin43 were detected between 43 and 47 kDa (1.0 ± 0.15; Figure 5). After COC were cultured in the medium with LH + FSH for 28 h, significant reductions in the intensity of the connexin43 bands were noted (0.27 ± 0.16; Figure 5). However, the addition of AGT to the gonadotrophin-containing medium produced a significant dose-dependent increase in the intensity of connexin43 bands (0.2×10^{-3} mol/l, 0.76 ± 0.31; 0.5×10^{-3} mol/l, 1.72 ± 0.25; Figure 5). Further addition of E_2 to the medium with AGT could not decrease the level of connexin43 in cumulus cells (1.60 ± 0.36), whereas the addition of progesterone to the medium did overcome the inhibitory effects of AGT on the level of connexin43 in the cumulus cells (0.27 ± 0.15, Figure 5). The intensity level of connexin43 bands in cumulus cells from COC cultured in the LH + FSH-containing medium with both progesterone and AGT was similar to that of

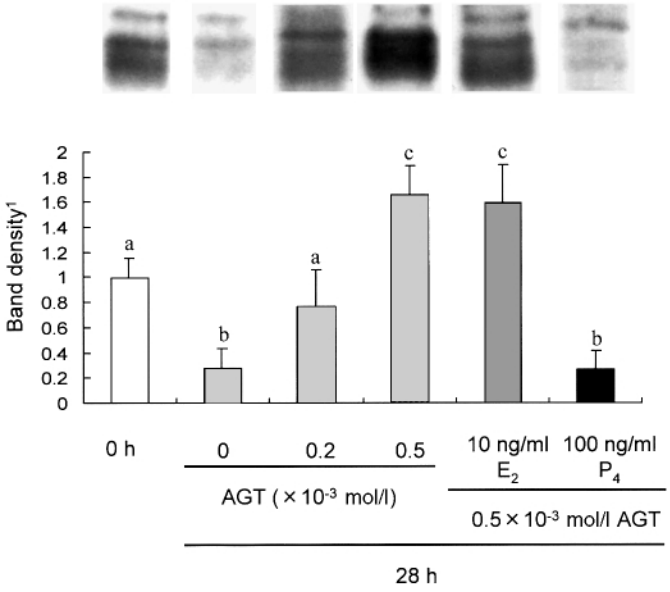

Figure 5. Effects of addition of aminoglutethimide (AGT) with or without steroids on gonadotrophin-suppressed connexin43 in cumulus cells surrounding oocytes cultured for 28 h. Connexin43 expression was assessed by immunoblot analysis. [1]Total density of connexin43 bands was expressed as the fold strength of the intensity in the cumulus cells just after collection from their follicles. [a–c]Columns with no common superscripts were significantly different ($P < 0.05$). E_2 = estradiol-17β; P_4 = progesterone.

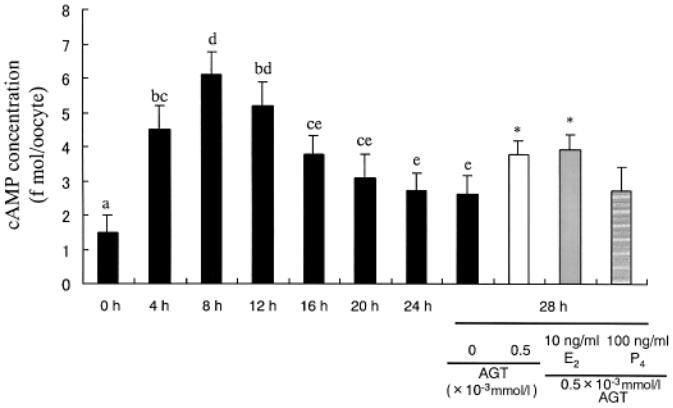

Figure 6. Time-dependent changes in cAMP level in oocytes surrounded with cumulus cells after culture in medium with LH + FSH, and effects of addition of aminoglutethimide (AGT) into the medium on cAMP contents of oocytes at 28 h cultivation. [a–e]Columns with common superscripts within time-dependent data on cAMP concentration were significant ($P < 0.05$). *Significant difference as compared with cAMP concentration in oocytes after culture for 28 h without AGT or progesterone ($P < 0.05$). E_2 = estradiol-17β; P_4 = progesterone.

cumulus cells isolated from COC cultured in the medium which lacked both AGT and progesterone (Figure 5).

The level of cAMP in the oocyte (Figure 6) immediately after recovery from the follicle was 1.5 ± 0.5 fmol, and 4 h cultivation of COC in the presence of LH + FSH produced a significant increase in the cAMP level in the oocyte. The concentration of cAMP reached a peak at 8 h of cultivation with LH + FSH, but it dramatically decreased after 12 h cultivation. An additional 16 h cultivation (i.e. a total of 28 h) of COC in the presence of FSH + LH significantly reduced the level of cAMP in oocytes (2.7 ± 0.5 fmol). However, cAMP levels in oocytes cultured for 28 h with 0.5×10^{-3} mol/l AGT and FSH + LH were significantly higher (3.8 ± 0.4 fmol) than that of oocytes cultured without AGT. This significant increase in cAMP concentration in the oocytes was abolished by the addition of

progesterone (2.7 ± 0.7 fmol), but not by addition of E_2 (3.9 ± 0.4 fmol). There was no significant difference in cAMP levels between oocytes cultured in the medium with LH+FSH alone, and with LH + FSH and AGT and progesterone.

Discussion

In human and rat follicles, PR has not yet been located in the granulosa cells of pre-ovulatory follicles before the endogenous LH surge, whereas PR mRNA levels in the cells are stimulated by exposure to ovulatory levels of LH (Park and Mayo, 1991; Revelli *et al*., 1996). In an in-vitro rat granulosa cell culture system (Park-Sarge and Mayo, 1994), it was shown that the expression of PR mRNA is evoked by both LH and FSH. LH and/or FSH have also been shown to cause an increase in progesterone production in the cumulus cells of pigs (Xia *et al*., 1994; Coskun *et al*., 1995), mice (Vanderhyden and Tonary, 1995), cattle (Armstrong *et al*., 1996) and humans (Chian *et al*., 1999). In the present study, expression was PR was not detected in the cumulus cells of COC immediately recovered from their follicles; however, numerous intense signals for PR immunoreactivity were observed in all layers of cumulus cells surrounding oocytes after culture with LH and FSH for 28 h. It was also demonstrated that significantly increased concentrations of progesterone were observed in the medium in which COC were cultured with LH and FSH for 28 h. Thus, these results provide the first evidence that during in-vitro meiotic maturation of porcine COC, LH and FSH induce PR production in cumulus cells; this change is concomitant with increased progesterone production.

To assess the role of progesterone in the meiotic resumption of porcine COC, a progesterone synthesis inhibitor, AGT, was administered. This condition included a supplementation to the medium containing gonadotrophins. When COC were cultured for 28 h in a gonadotrophin-containing medium supplemented with 0.5×10^{-3} mol/l AGT, the PR was still synthesized in cumulus cells but an almost complete inhibition of both progesterone production and GVBD was seen. However, this inhibitory effect on GVBD was overcome by further addition of progesterone into the medium. These results suggest that the progesterone secreted by LH- and/or FSH-stimulated cumulus cells activates the de-novo-synthesized PR in the cumulus cells, and induces GVBD. This is supported by the observation that, in the absence of LH and FSH, progesterone was unable to affect the rate of GVBD in the porcine oocytes.

One of the known roles of progesterone is the down-regulation of gap junctional communication in human myometrial cells (Zhao *et al*., 1996). This down-regulated communication results in part from the reduction of connexin43 mRNA expression, as caused by the PR-mediated transcription pathway in the rat endometrium (Grummer *et al*., 1994). In the present study, significant reductions in the intensity of connexin43 bands were noted in the cumulus cells surrounding oocytes cultured for 28 h in gonadotrophin-containing medium; however, the addition of AGT into the medium led to a high intensity of connexin43 bands in the cumulus cells after 28 h cultivation. This is consistent with the finding that in porcine COC, the total amount of connexin43 in cumulus cells is markedly reduced after 12 h of culture, concomitant with re-initiation of meiosis (Shimada *et al*., 2001). At this time point, the progesterone synthesis stimulated by the addition of FSH and LH to the maturation medium (22.1 ± 2.3 ng/ml) is significantly increased when compared to the control (3.0 ± 0.4 ng/ml) (unpublished data). One study reported that the LH-induced gating mechanism of the gap junctions in rat ovarian follicles is manifested by a reduction of connexin43 protein levels, due to attenuation of gene expression (Granot and Dekel, 1994). Taken together, the data imply that in cumulus cells surrounding porcine oocytes, the secreted progesterone into the medium causes a reduction of connexin43 synthesis through the PR-mediated signal transduction pathway.

Furthermore, in mouse oocytes, a drop in oocyte cAMP level has been shown to be involved in resumption of meiosis (Schultz *et al*., 1983). It was revealed (Yoshimura *et al*., 1992) that a transient increase followed by a decrease in cAMP level induced meiotic resumption of rabbit oocytes. In gilts treated with PMSG and HCG, the cAMP concentration in oocytes increases transiently after HCG injection; then the concentration returns to a low level and this reduction is accompanied by the initiation of GVBD (Mattioli *et al*., 1994). In this study, the cAMP level in porcine oocytes surrounded with cumulus cells was increased by the stimulation of FSH and LH, and the maximal level was recognized at 8 h culture. Further time in culture induced a significant decrease in the level of cAMP, concomitant with GVBD in porcine oocytes. The present study also showed that the cAMP level in oocytes cultured with AGT was significantly higher than that of oocytes cultured without AGT or that of oocytes cultured with both AGT and progesterone. Furthermore, in our previous study (data not shown), the addition of IBMX was shown to induce an increase in cAMP levels in oocytes at the same level as that in oocytes cultured with AGT, and to suppress GVBD in the oocytes. It has been reported that cAMP synthesized in cumulus cells may be transferred to oocytes via gap junctional communications (Schultz *et al*., 1983). Additionally, in this study we showed that progesterone is associated with a reduction of connexin43 in cumulus cells. These findings support our hypothesis that the secreted progesterone mediates the close of gap junctional communication, leading to GVBD in porcine COC.

In summary, PR were not detected in cumulus cells of COC either immediately recovered from their follicles or when cultured for 28 h without LH and FSH. However, LH and FSH induced PR production in cumulus cells, and this was associated with increased progesterone production. The addition of AGT into gonadotrophin-containing medium caused low levels of progesterone in the medium, a high intensity of connexin43 bands in the cumulus cells, a suppression of a decrease in cAMP level in oocytes, as well as a rise in the proportion of oocytes arrested at the GV stage after a 28 h cultivation. Thus, we suggest that the binding of progesterone secreted by LH- and FSH-stimulated cumulus cells to its newly synthesized receptor induces GVBD in porcine oocytes, presumably through the close of gap junctional communication between cumulus cells.

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