Time Dependent Changes in Progesterone Receptor Expression in Cumulus Cells During Meiotic Resumption of Porcine Oocytes

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Abstract: *In this study, to investigate the time dependent changes in progesterone receptor (PR) expression in cumulus cells during meiotic resumption of porcine oocytes, each amount of PR-A and PR-B mRNA was analyzed by RT-PCR with primer sets for the PR-B region and the PR-A/B common region. The results showed that the levels of both PR-A/B and PR-B mRNA were very low in cumulus cells immediately recovered from their follicles. The cultivation with FSH and LH significantly increased the level of both PR-A/B and PR-B mRNA in cumulus cell of COCs, whereas the level of PR-B mRNA significantly decreased at 12-hr cultivation. Nevertheless, the higher level of PR-A/B mRNA was maintained up to 20-hr cultivation, suggesting that PR-A was mainly expressed in cumulus cells during cultivation from 12 hr to 20 hr. When COCs were cultured for 10 hr and then further cultured with RU486 for 10 hr, the proportion of oocytes undergoing GVBD significantly decreased in a dose dependent fashion. These results suggest that the high ratios of PR-A to PR-B in cumulus cells of COCs during 12-hr to 20-hr cultivation, are required for meiotic resumption of porcine cumulus-enclosed oocytes in vitro.*

Key words: *Progesterone receptor, Cumulus cells, Oocytes, Meiotic resumption, IVM*

Introduction

During *in vitro* meiotic maturation of cumulus-oocyte complexes (COCs), progesterone was produced by cumulus cells, and the level of progesterone was increased by stimulation with LH, FSH or forskolin in humans [1], rats [2], cattle [3] and pigs [4–6]. Morgan *et al*. [7] reported that a high concentration of progesterone in follicular fluid induced meiotic maturation of rhesus monkey oocytes. In our previous study [8], we showed that there was a significant positive correlation between the GVBD rate of oocytes and the progesterone concentration in each well. When porcine COCs were cultured with P450scc inhibitor, progesterone production was almost completely suppressed in cumulus cells, and a reduction in the GVBD rate was also observed [6]. Therefore, progesterone secreted by cumulus cells is associated with meiotic maturation of porcine oocytes.

Many of the biological activities of progesterone were mediated by an intracellular receptor, a hormonally regulated DNA-binding protein that belongs to a superfamily of ligand-activated transcription factors [9, 10]. The progesterone receptor (PR) was expressed as two protein isoforms, PR-A and PR-B, that were produced from a single gene by transcription at two distinct promoters and by translation initiation by two alternative AUG signals [11, 12]. They also showed that PR-A was a truncated form of PR-B that lacked the first 164 N-terminal amino acids [11, 12]. Two PR isoforms from the PR gene were also observed in porcine granulosa cells [13]. In PR-A knock-out mice, Mulac-Jericevic *et al.* [14] showed that PR-A and PR-B were functionally distinct mediators of progesterone action in uterine epithelium. Nevertheless, there is little information on the ratios of PR-A to PR-B mRNA, and the functional differences between PR-A and PR-B in cumulus cells during meiotic resumption of porcine oocytes.

In this study, we examined the time dependent

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changes in PR expression in cumulus cells of COCs. Each amount of PR-A and PR-B mRNA was analyzed by RT-PCR with the primer sets for the PR-B region and PR-A/B common region.

Materials and Methods

In vitro maturation of porcine COCs

Isolation of porcine COCs was described previously [15]. The COCs were cultured in the maturation medium supplemented with 20 ng/ml highly purified porcine FSH (NIDDK, Torrance, CA, USA) and 1.0 μg/ml highly purified porcine LH (NIDDK). The maturation medium was modified NCSU37 [16] supplemented with 10% (v/v) FCS (Gibco BRL, Grand Island, NY, USA), 7 mM Taurine (Sigma Chemical Co., St Louis, MO, USA) and 2 mM hypoxanthine (Sigma). The oocytes were fixed with acetic acid/ethanol (1:3) for 48 hr, and stained with aceto-lacmoid before examination under a phase-contrast microscope (400 $\times$) for evaluation of their chromatin configuration.

RNA isolation

At the end of cultivation, cumulus cells were separated from 20 COCs by pipetting with flame-draw pipette tips, whose inner diameters were slightly larger than the oocyte diameter. After cumulus cells were washed with PBS three times, total RNA was extracted from cumulus cells using by means of an SV Total RNA Isolation System (Promega, Madison, WI, USA), according to the instruction manual, and dissolved in nuclease-free water. The final RNA concentrations were determined by absorbance with a spectrophotometer.

RT-PCR

RT-PCR was performed according to our previous study [17]. Briefly, 10 ng of total RNA was reverse transcribed at 48 degrees C for 45 min, denatured at 94 degrees C for 2 min, and amplified for 33 cycles of denaturation at 94 degrees C for 30 sec, primer annealing at 59 degrees C (PR-B) or 56 degrees C (PR-A/B, β-actin) for 1 min, and extension at 68 degrees C for 1 min, with a final extension step of 7 min at 68 degrees C. The amplified products were analyzed by electrophoresis on 2% agarose gels. One primer set was directed at the sequence specific for the PR-B (*i.e.* within the 164 amino acids at the N-terminus) and therefore detected only mRNA transcripts encoding PR-B (Fig. 1). The other primer set was directed to the section of the PR common to PR-A and PR-B (PR-A/B) and therefore detected total PR mRNA (Fig. 1). β-actin was used as a control for reaction efficiency and variations in concentrations of mRNA in the original RT reaction. The amplified product was electrophoresed on 2 % (w/v) agarose gel and visualized by ethidium bromide staining. The intensity of the objective bands was quantified by densitometric scanning with a Gel-Pro Analyzer (Media Cybernetics, MD, USA). The respective values of PR-A/B and PR-B were normalized according to those of β-actin to evaluate arbitrary units of the relative abundance of the targets.

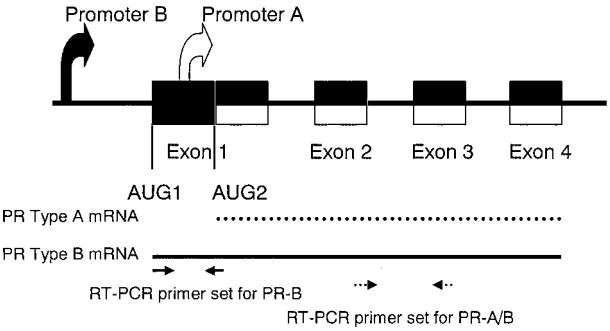

Fig. 1. Schematic diagram indicating positions of the oligonucleotide primers on the putative porcine PR gene. Although unknown, the genomic structure of the porcine PR is depicted in a similar fashion to the human PR gene (DDBJ, Accession Number, NM 000926).

Treatment of COCs with RU486

COCs were pre-cultured with 20 ng/ml FSH + 1.0 μg/ml LH for 10 hr and then further cultured with FSH, LH and either 0.25 or 2.5 μM RU486 for 10 hr. RU486 (Sigma) was dissolved in ethanol at 25 mM, and the final concentration of each was obtained by dilution in the maturation medium.

Statistical analysis

Statistical analyses of all data from three or four replicates for comparison were carried out by one-way ANOVA followed by Duncan's multiple-range test (Statview; Abacus Concepts, Inc., Berkeley, CA). All percentage data were subjected to arcsine transformation before analysis. Differences were considered significant when $P<0.05$.

Results and Discussion

Our data showed that the level of total PR mRNA (PR-A/B) was very low in cumulus cells just after

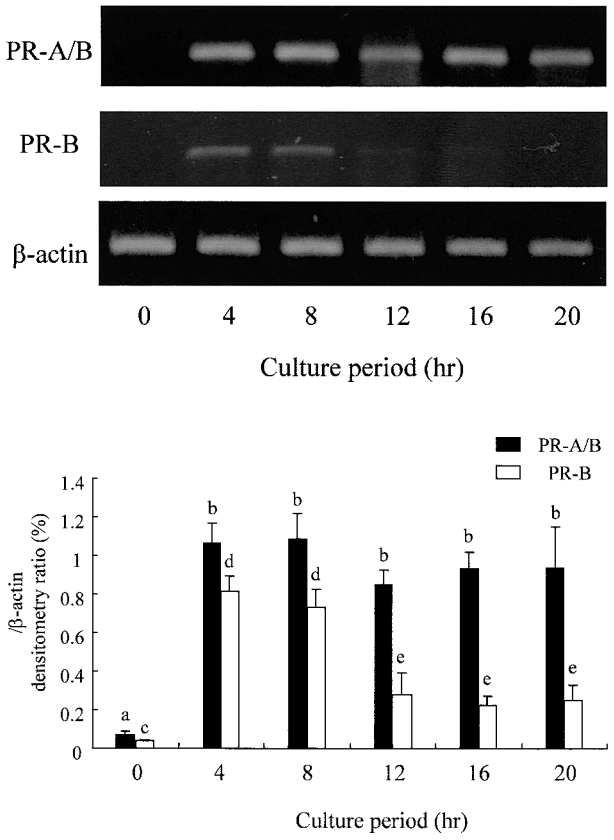

Fig. 2. Time dependent changes in PR expression in cumulus cells of COCs which were cultured with FSH and LH. [a-b, c-e:] Ratios with different letters were significant ($p<0.05$). Values are the mean ± SEM of 3 replicates.

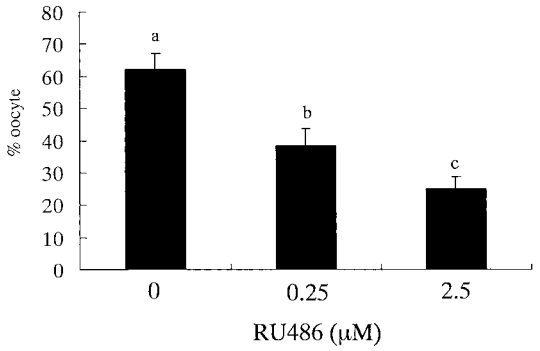

Fig. 3. The effects of RU486 on the proportion of oocytes undergoing GVBD when COCs were cultured for 10 hr with FSH and LH and then further cultured for 10 hr with additional 0.25 or 2.5 μM RU486. [a-c]: Ratios with different letters were significant ($p<0.05$). Values are the mean ± SEM of 3 replicates.

collection from their follicles (Fig. 2), but the level was significantly increased by 4-hr cultivation with FSH and LH, and the higher level was maintained up to 20-hr cultivation ($P<0.05$) (Fig. 2). Within the 5' flanking regions of the PR gene, two putative functional promoters have been described [11]. The distal and proximal promoters had putative binding sites for the estrogen receptor, designated estrogen response element (ERE)-like regions [11, 12]. In MCF-7 breast cancer cells, stimulation with estradiol 17β induced the expression of the PR gene [18], but in granulosa cells, expression of the PR gene is not observed on stimulation with estrogen, and the cAMP-PKA pathway has been documented to directly activate the distal promoter activity of the PR gene in the cells [19]. The level of cAMP in cumulus cells was increased by FSH and LH [20]. These results suggested that expression of the PR gene was stimulated by the cAMP-PKA pathway in cumulus cells as well as in granulosa cells.

In the present study, the expression of PR-B in cumulus cells was increased by FSH and LH during the first 8-hr cultivation, but the level was significantly decreased at 12-hr cultivation ($P<0.05$) (Fig. 2). Nevertheless, the higher level of PR-A/B mRNA was maintained up to 20-hr cultivation (Fig. 2), suggesting that PR-A was mainly expressed in cumulus cells during cultivation from 12 hr to 20 hr. In our previous study [21], the progesterone level in the medium where porcine COCs had been cultured for 10 hr was low (11.5 ± 2.1 ng/ml), whereas a significantly higher level was detected in the medium after COCs had been cultured for 20 hr (28.5 ± 3.3 ng/ml). Moreover, we also showed that the progesterone secreted by cumulus cells played an important role in the loss of gap junctional communication in cumulus cells layers after 12-hr cultivation of porcine COCs [6]. Therefore, it was estimated that the binding of progesterone to the newly synthesized PR-A in cumulus cells was involved in the meiotic resumption of cumulus-enclosed oocytes.

To investigate whether the binding of progesterone to PR-A in cumulus cells induced the meiotic resumption of oocytes, COCs were cultured with progesterone receptor antagonist RU486 after 10-hr cultivation of COCs with FSH and LH. As shown in Fig. 3, the proportion of oocytes undergoing GVBD was significantly decreased as the concentration of RU486 increased in medium with FSH and LH ($P<0.05$). It has been reported that the administration of anti-progestin antiserum to PMSG+hCG treated female rats significantly decreased the incidence of meiotic

resumption of oocytes within the large follicles [22]. Osborn *et al.* [23] also reported that when ovine follicles were cultured with FSH, LH and P450scc inhibitor, aminoglutethimide (AGT), the result was an almost complete inhibition of progesterone production and meiotic progression to the metaphase II stage in oocytes. Moreover, in our previous study [6], during *in vitro* maturation of porcine COCs, the addition of AGT significantly suppressed both progesterone production by COCs and GVBD in cumulus-enclosed oocytes. These previous reports have indicated that progesterone is required for the meiotic resumption of oocytes, but the present study is the first to demonstrate that the PR expression pattern in porcine cumulus cells shifts from PR-B to PR-A; the high ratios of PR-A to PR-B in cumulus cells induce the meiotic resumption of cumulus-enclosed oocytes.

In conclusion, expression of the PR gene in cumulus cells was stimulated by FSH and LH. During the first 8-hr cultivation periods, a higher level of both PR-A/B and PR-B mRNA was detected in cumulus cells of COCs, whereas the level of PR-B mRNA was significantly decreased at 12-hr cultivation. During the 12-hr to 20-hr cultivation, PR-A was mainly expressed in cumulus cells. When COCs were cultured for 10 hr and then further cultured with RU486 for 10 hr, the proportion of oocytes undergoing GVBD was significantly decreased in a dose dependent fashion. These results suggest that the high ratios of PR-A to PR-B in cumulus cells during the 12-hr to 20-hr cultivation, play an important role in meiotic resumption of porcine cumulus-enclosed oocytes *in vitro*.

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