Distributional Changes of Lysosomes in In Vitro Matured and Fertilized Porcine Oocytes Visualized by Acridine Orange Staining

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Abstract: *The distribution of lysosomes during porcine oocyte maturation and fertilization in vitro was revealed by using acridine orange staining. The lysosomal distribution was classified into three types: generally even distribution throughout the ooplasm (Type I), less distribution in the peripheral ooplasm (Type II), and less distribution in the peripheral and inner ooplasm (Type III). All oocytes examined at 0 and 8 h after maturation culture showed the Type I lysosome distribution and 97% were at the germinal vesicle (GV) stage. When cultured for 22 h, the relative abundance of Type I oocytes decreased to 58%, while Type II and Type III oocytes appeared at relative abundances of 35 and 8%, respectively. After 32 and 44 h of culture, 35 and 80% of the oocytes, respectively, were Type III. When cultured with olomoucine, IBMX or dbcAMP for 22 h, 100, 79 and 94% of the oocytes, respectively, showed the Type I distribution of lysosomes, and their nuclei were almost all at the GV stage (100, 93 and 100%). The results of the present study suggest that there may be a close relationship between nuclear maturation and the distribution of lysosomes in the cytoplasm, and that the distribution of lysosomes may be one of the criteria of cytoplasmic maturation of porcine oocytes.*

Key words: *Porcine oocyte, Maturation, Fertilization, Lysosome, Acridine orange*

Introduction

Lysosomes are membrane-bounded organelles, found in the cytoplasm of eukaryotic cells, which contain digestive enzymes. They are usually investigated in subcellular fractions and in preparations of fixed cells. Recently, the distribution of lysosomes in the oocyte has been investigated using acridine orange (3, 6-bis-dimethylaminoacridine, AO) staining method in rats [1–3] and hamsters [4]. In these animals, lysosomes are distributed throughout the cytoplasm of the germinal vesicle (GV) oocytes, but just before GV breakdown they temporarily move to the perinuclear region and then rapidly spread again throughout the entire cytoplasm after GV breakdown [1–4]. Therefore, the distributional change of lysosomes is suggested to be related to nuclear maturation in oocytes [1–4]. However, observations of the oocytes of domestic animals have not yet been reported.

In the present study, the distribution of lysosomes was examined in porcine oocytes in the process of maturation and fertlization *in vitro* using the AO staining method. We also investigated the distribution of lysosomes in oocytes treated with 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (olomoucine), 3-isobutyl-1- methylxanthine (IBMX) or dibutyryl cAMP (dbcAMP) in order to clarify the relationship between nuclear maturation and the translocation of lysosomes in the cytoplasm. Olomoucine has an inhibitory effect on the activity of $p34^{cdc2}$, a cyclin dependent kinase of MPF [5, 6]. On the other hand, IBMX is known to be an inhibitor of cAMP phosphodiesterase, which metabolizes cAMP to 5'-AMP, and acts to maintain cAMP at a higher level in the cytoplasm [7, 8]. It has been confirmed that maintaining cAMP at a higher level by treatment with IBMX [9, 10] or dbcAMP [11, 12], an analogue of cAMP, results in the suppression of the resumption of maturation division in mammalian oocytes.

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Materials and Methods

Collection and culture of COCs

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl solution maintained at 37°C. The ovaries were washed in 0.9% NaCl solution containing 200 i.u./ml potassium penicillin G. Immature oocytes covered with cumulus cells (COCs) were aspirated from medium-sized follicles (3–6 mm in diameter) with a 21-gauge needle fixed to a 10-ml disposable syringe. Collected COCs were washed in PBS (pH 7.4) [13] and then in a culture medium composed of TCM-199 (Gibco BRL, NY, USA) supplemented with 10% (v/v) porcine follicular fluid, 10% (v/v) fetal calf serum (FCS; Gibco BRL), 10 i.u./ml eCG (PEAMEX; Sankyo Yell Yakuhin Co. Ltd., Tokyo, Japan), 10 i.u./ml hCG (Gonatropin; Teikoku Hormone Manufacturing Co. Ltd., Tokyo, Japan) and 0.001% (w/v) estradiol-17β (Wako Pure Chemical Industries, Osaka, Japan) [14]. Groups of 40 to 50 COCs were transferred into each well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 400 μl/well of the culture medium, which had previously been covered with mineral oil (Sigma-Aldrich Inc., MO, USA) and equilibrated in a CO_2 incubator (Asahi Life Science, Tokyo, Japan). For maturation, these COCs were cultured at 39°C in an atmosphere of 5% CO_2 in air.

In vitro fertilization of oocytes

Ejaculated boar semen was treated by the method of Wang *et al.* [15], in order to induce capacitation of spermatozoa. The semen was washed three times in Brackett and Oliphant (BO) [16] medium containing 5 mM caffeine (Sigma-Aldrich Inc.) and 0.3% bovine serum albumin (BSA; Sigma-Aldrich Inc.). Spermatozoa were resuspended in BO medium containing 5 mM caffeine and 0.3% BSA to give a concentration of 5×10^5 live spermatozoa/ml, and 400 μl of sperm suspension was covered with mineral oil in each well of a Nunc 4-well multidish.

At 44 h after maturation culture, the COCs were washed twice in BO medium containing 5 mM caffeine and 0.3% BSA. Forty to 50 COCs were introduced into the sperm suspension and cultured at 39°C in a CO_2 incubator (5% CO_2 in air). After culture with spermatozoa for 6 h, the COCs were washed twice in PBS, and then the cumulus cells were dispersed from the oocytes by pipetting in PBS containing 0.1% hyaluronidase (Sigma-Aldrich Inc.). The denuded oocytes were washed and cultured in NCSU 37 containing 2.73 mM sodium lactate, 0.17 mM sodium pyruvate and 0.4% BSA [17] at 39°C in a CO_2 incubator (5% CO_2 in air).

Observations of lysosome distribution and nuclear maturation

At 0, 8, 22, 32 and 44 h after maturation culture, the COCs were washed in Earle's balanced salt solution (EBSS, pH 7.3) [18], and the cumulus cells surrounding the oocytes were dispersed by pipetting in EBSS containing 0.1% hyaluronidase (Sigma-Aldrich Inc.). These denuded oocytes and the oocytes obtained at 6, 12, 24 and 32 h after insemination were washed in EBSS containing 25 mM HEPES (Wako Pure Chemical Industries) and 0.3% BSA (mEBSS), and further immersed in mEBSS containing 0.2% pronase (Sigma-Aldrich Inc.), to dissolve the zona pellucida. These zona-free oocytes were washed three times in mEBSS, and then immersed in a staining solution composed of 500 μg AO (MERCK, Dermstadt, Germany) and 100 ml mEBSS for 5 min at room temperature. After staining, the oocytes were washed three times in mEBSS and placed on glass slides to be photographed under a reflected-light fluorescence microscope (Nikon Corporation, Tokyo, Japan). The same staining procedures were repeated three times for each culture period. Degenerated oocytes were eliminated from the observation.

After observation of the lysosomes, the oocytes were fixed in 25% (v/v) acetic acid in ethanol for 48 h at room temperature and stained with 1.0% aceto-orcein to examine the nuclear configuration.

Effects of inhibitors on lysosome distribution

COCs were cultured for 22 h at 39°C in the culture medium containing 400 μM olomoucine (Sigma-Aldrich Inc.), 500 μM IBMX (Sigma-Aldrich Inc.) or 2.0 mM dbcAMP (Sigma-Aldrich Inc.). Olomoucine and IBMX were previously dissolved in dimethyl sulfoxide (DMSO) and then diluted with the culture medium to 400 and 500 μM, respectively. The concentration of DMSO in the culture medium was 0.37% (v/v) for olomoucine and 0.1% (v/v) for IBMX. dbcAMP was directly dissolved in the medium. COCs cultured for 22 h in the medium without inhibitor were used as controls. Some oocytes cultured with inhibitors were subsequently cultured in the inhibitor-free medium to observe translocation of lysosomes. Thereafter, the nuclear configurations were examined as described above.

Statistical analysis

The rates of nuclear maturation and the number of

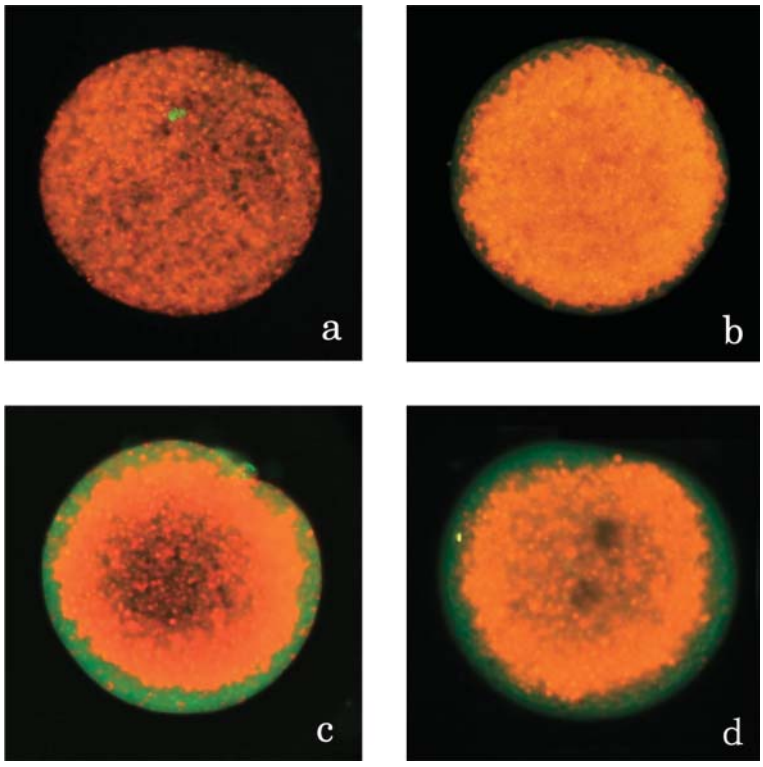

Fig. 1. Fluorescent micrographs of porcine oocytes stained with acridine orange. a. A Type I oocyte at the germinal vesicle stage just after collection. Lysosomes are distributed throughout the cytoplasm. b. A Type II oocyte 22 h after maturation culture. Lysosomes are distributed in the cytoplasm except for the cortical region. c. A Type III oocyte 44 h after maturation culture. Lysosomes are distributed in the cytoplasm except for the cortical region, and their density is lower in the inner cytoplasm. d. An oocyte 24 h after insemination. Lysosomes are distributed in the cytoplasm except for the cortical region, and their density is lower in the inner cytoplasm.

oocytes with different distribution patterns of lysosomes were statistically analyzed by the Chi-square test.

Results

Lysosome distribution and meiotic stages in the oocytes during in vitro maturation

Distribution patterns of AO-stained lysosomes in porcine oocytes were classified into three types. In Type I, the lysosomes were distributed throughout the cytoplasm (Fig. 1a). In Type II, the lysosomes were distributed in the cytoplasm except for the cortical region (Fig. 1b). In Type III, the lysosomes were distributed in the cytoplasm except for the cortical region, and their density was lower in the inner cytoplasm (Fig. 1c).

Immediately after collection (0 h) and at 8 h after culture, all oocytes showed Type I lysosome distribution (Table 1). The majority of these oocytes were at the GV stage (97 and 83% at 0 and 8 h, respectively). At 22 h after culture, the relative abundance of Type I oocytes had decreased to 58%, while Type II and Type III oocytes had appeared at the relative abundances of 35 and 8%, respectively. The Type II oocytes were at the diakinesis, metaphase I and anaphase I stages. Among the oocytes cultured for 32 and 44 h, 35 and 80% were Type III, respectively, and the nuclei of the Type III oocytes were at the metaphase I to metaphase II stages.

As shown in Fig. 2, the lysosome distribution changed from Type I at the GV stage to Type III at the metaphase II stage during oocyte maturation. Type II was considered to be an intermediate phase between Type I and Type III.

Table 1. Changes in lysosome distribution of porcine oocytes matured *in vitro*

Hours of culture	No. of oocytes examined	No. and (%) of oocytes with different types* of lysosome distribution		
		I	II	III
0	38	38 (100)[a]	0 (0)[c]	0 (0)[c]
8	35	35 (100)[a]	0 (0)[c]	0 (0)[c]
22	40	23 (58)[b]	14 (35)[a]	3 (8)[c]
32	51	7 (14)[c]	26 (51)[a]	18 (35)[b]
44	49	3 (6)[c]	7 (14)[b]	39 (80)[a]

*Type I: Lysosomes were distributed throughout the cytoplasm. Type II: Lysosomes were distributed in the cytoplasm except for the cortical region. Type III: Lysosomes were distributed in the cytoplasm except for the cortical region, and their density was lower in the inner cytoplasm. Values with different superscripts in the same column are significantly different ($P < 0.05$).

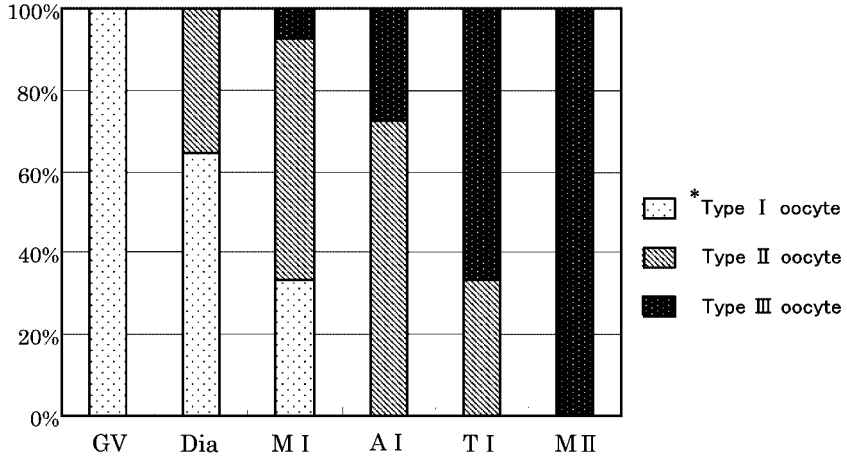

Fig. 2. Different types of lysosome distribution in the porcine oocytes at the stages of germinal vesicle (GV), diakinesis (Dia), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII). *Type I: Lysosomes were distributed throughout the cytoplasm. Type II: Lysosomes were distributed in the cytoplasm except for the cortical region. Type III: Lysosomes were distributed in the cytoplasm except for the cortical region, and their density was lower in the inner cytoplasm.

Lysosome distribution and meiotic stages in the oocytes treated with olomoucine, IBMX or dbcAMP

The lysosome distributions and nuclei of porcine oocytes treated with olomoucine, IBMX or dbcAMP are shown in Table 2. All the oocytes cultured for 22 h in the medium containing olomoucine showed the Type I distribution of lysosomes and remained at the GV stage. Among the oocytes treated with IBMX or dbcAMP, 79 and 94%, respectively, showed the Type I distribution of lysosomes, and almost all oocytes were at the GV stage (93 and 100% for IBMX and dbcAMP, respectively). The nuclei of control oocytes cultured without inhibitors were beyond the metaphase I stage. Therefore, these results confirm that treatment with olomoucine, IBMX or dbcAMP inhibits not only the resumption of nuclear maturation in porcine oocytes but also the distributional change of lysosomes in their cytoplasm.

When the inhibitor-treated oocytes were transferred into inhibitor-free medium and cultured for an additional 22 h, more than half of them reached the metaphase II stage (60, 63 and 60% for olomoucine, IBMX and dbcAMP, respectively). Of these treated oocytes, 88 (35/40), 100 (40/40) and 100% (48/48), respectively, showed Type II and Type III distributions of lysosomes. No significant differences were noted compared to the maturation rates (80, 74 and 77%) and the percentages of the Type II and Type III lysosome distributions (96, 90 and 96%) of control oocytes. These results suggest that the abilities of nuclear maturation and lysosome movement of the treated oocytes were sustained.

Table 2. Changes in lysosome distribution and nuclear maturation of porcine oocytes cultured with olomoucine, IBMX or dbcAMP

Treatments	No. of oocytes examined	Meiotic stages		No. and (%) of oocytes with different types* of lysosome distribution		
		Germinal vesicle ≤	Diakinesis	I	II	III
Olomoucine	31	31 (100)[a]	0 (0)[b]	31 (100)[a]	0 (0)[b]	0 (0)[a]
None (Control)	24	5 (21)[b]	19 (79)[a]	9 (38)[b]	13 (54)[a]	2 (8)[a]
IBMX	28	26 (93)[a]	2 (7)[b]	22 (79)[a]	6 (21)[a]	0 (0)[a]
None (Control)	29	12 (41)[b]	17 (59)[a]	14 (48)[b]	13 (45)[a]	2 (7)[a]
dbcAMP	32	32 (100)[a]	0 (0)[b]	30 (94)[a]	2 (6)[b]	0 (0)[a]
None (Control)	34	9 (26)[b]	25 (74)[a]	20 (59)[b]	11 (32)[a]	3 (9)[a]

The oocytes were observed after 22 h of culture. *Type I: Lysosomes were distributed throughout the cytoplasm. Type II: Lysosomes were distributed in the cytoplasm except for the cortical region. Type III: Lysosomes were distributed in the cytoplasm except for the cortical region, and their density was lower in the inner cytoplasm. Values with different superscripts in the same column in each experimental lot are significantly different ($P < 0.05$).

Lysosome distribution in the oocytes after fertilization

At 6 h after insemination, the Type III distribution of lysosomes was observed (Fig. 1d). This distributional pattern of lysosomes in sperm-penetrated oocytes remained unchanged until the pronuclear stage at 32 h after insemination.

Discussion

The present study revealed that AO-stained lysosomes were distributed throughout the cytoplasm at the GV stage of porcine oocytes (Type I), and that lysosomes disappeared from the cortical cytoplasm (Type II), and then reduced their density in the inner cytoplasm (Type III) as nuclear maturation progressed. Olomoucine, IBMX or dbcAMP inhibited the resumption of nuclear maturation and the translocation of lysosomes in the cytoplasm. When oocytes cultured with these inhibitors were further cultured in the medium without inhibitors, both nuclear maturation and translocation of lysosomes progressed normally. These results suggest that changes in the lysosome distribution from the Type I to Type III may occur in association with the resumption of meiotic maturation, since accumulation of lysosomes around the nuclei prior to GV breakdown has been reported in rat [1–3] and hamster [4] oocytes. Furthermore, the present study showed that the distributional pattern of lysosomes after *in vitro* fertilization was Type III, as observed in the mature oocytes cultured for 44 h, suggesting that this pattern may remain unchanged until the first cleavage [19].

It has been reported that during nuclear maturation of porcine oocytes various changes occur in the cytoplasm, including accumulation of mitochondria in the inner cytoplasm [20], movement of endoplasmic reticula [21] and cortical granules [22–25] to the cytoplasm immediately beneath the plasma membrane, reduction in the size of lipid droplets [26], and reduction in the metabolic ability of some steroids [27]. Since the movement of the cortical granules [25], the reduction in the size of the lipid droplets [26] and the reduction in the steroid metabolism [27] in the cytoplasm were all inhibited in porcine oocytes treated with olomoucine, as well as the resumption of nuclear maturation, it has been suggested that nuclear maturation is closely associated with such changes in the cytoplasm [25–27]. The results of the present study suggest that there may be a close relationship between nuclear maturation and the distribution of lysosomes in the cytoplasm, and that the distribution of lysosomes may be one of the criteria of cytoplasmic maturation of porcine oocytes. However, the mechanism underlying the distributional changes of lysosomes in the cytoplasm in association with oocyte maturation remains unknown. This issue should be further studied.

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