Nuclear Maturation, Steroid Metabolism and Lipid Droplets in Porcine Oocytes Cultured with IBMX or dbcAMP

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(Received August 3, 2008)

Summary

We previously reported in porcine oocytes that the activities of some hydroxysteroid dehydrogenases (HSDs) and the size of lipid droplets in the cytoplasm decreased as nuclear maturation progressed, and that the progression of nuclear maturation, the decrease of HSD activities and the reduction in the size of lipid droplets did not occur in those treated with olomoucine. From these results, we suggested that the changes in the steroid metabolism and the size of lipid droplets in the cytoplasm were closely associated with nuclear maturation. However, whether olomoucine directly acts on the cytoplasm to inhibit such changes could not be determined. In the present investigation, the activities of some HSDs and Sudanophilic lipid droplets were histochemically demonstrated in porcine oocytes in which the nuclear maturation was suppressed and the high cAMP level in the cytoplasm was maintained by the treatment of IBMX or dbcAMP, in order to clarify the relationship between nuclear maturation and the metabolism of steroids and the number of lipid droplets in the cytoplasm.

Of the oocytes cultured with IBMX or dbcAMP for 22 hrs, 97 and 100 % were in the germinal vesicle (GV) stage, respectively. The percentages of oocytes in the GV stage were significantly higher in both the treated oocytes than in control oocytes. The rates of the treated oocytes showing the activities of Δ^5-3β-HSD (using pregnenolone and 17α-hydroxypregnenolone as the substrates), 17β-HSD (estradiol-17β), 20α-HSD (20α-hydroxyprogesterone) and 20β-HSD (17β-hydroxyprogesterone) did not differ from those of control oocytes. Also, there were no differences in the number of lipid droplets of different sizes between IBMX- or dbcAMP-treated oocytes and control oocytes.

From these findings, it was suggested that the changes in the steroid metabolism and the size of lipid droplets in the cytoplasm with oocyte maturation depend on the cAMP level in their cytoplasm rather than the progression of nuclear maturation.

Bull.Facul.Agric.Niigata Univ., 61(1):27–33, 2008

Key words : porcine oocyte, cAMP, steroid metabolism, lipid droplet, histochemistry

It is reported that together with the nuclear maturation of oocytes, various changes occur in the cytoplasm. In porcine oocytes, we have reported that the percentages of oocytes showing the activities of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) (using DHA as the substrate), 17β-HSD (testosterone) and 20β-HSD (20β-hydroxyprogesterone) did not change during maturation culture, while those showing the activities of Δ^5-3β-HSD (pregnenolone and 17α-hydroxypregnenolone), 17β-HSD (estradiol-17β), 20α-HSD (20α-hydroxyprogesterone) and 20β-HSD (17β-hydroxyprogesterone) decreased as nuclear maturation progressed (Takano and Niimura, 2002). We have also reported that the number of large lipid droplets decreased remarkably, while the number of small and medium ones increased in porcine oocytes as the nuclear maturation progressed (Niimura *et al*., 2002). Furthermore, we have observed the activities of HSDs and the number of lipid droplets in porcine oocytes treated with 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (olomoucine), which was known to be an inhibitor of the activity of $p34^{cdc2}$, a cyclin dependent kinase of MPF (Vesely *et al*., 1994; Abraham *et al*., 1995), in order to determine the relationship between nuclear maturation and changes in the steroid metabolism and the number of lipid droplets in the cytoplasm. The resumption of nuclear maturation was completely inhibited in olomoucine-treated oocytes, and the decrease of the activities of HSDs and the reduction in the size of lipid droplets were also inhibited (Niimura *et al*., 2002; Takano and Niimura, 2002). From these findings, we suggested that the changes in the metabolism of some steroids and the size of lipid droplets in the cytoplasm are closely associated with nuclear maturation (Niimura *et al*., 2002; Takano and Niimura, 2002). However, whether olomoucine directly acts on the cytoplasm to inhibit such changes in steroid metabolism and size of lipid droplets could not be determined.

It is generally known that cAMP synthesized in cumulus cells by the stimulation of LH flows into the cytoplasm of oocytes through gap junctions between cumulus cells and oocytes, and plays some important roles in oocyte maturation (Petr *et al*., 1991; Mattioli *et al*., 1994; Funahashi *et al*., 1997; Shimada and Terada, 2002; Shimada *et al*., 2002). Recently, we have reported that the amount of cAMP in porcine cumulus-

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Table 1. HSDs investigated, and substrates, solvents and cofactors used for their histochemical analyses

HSDs	Substrates	Solvents	Cofactors
Δ^5-3β-HSD	Pregnenolone	Acetone	NAD
	17α-Hydroxypregnenolone	Dimethylformamide	NAD
17β-HSD	Estradiol-17β	Acetone	NAD
20α-HSD	20α-Hydroxyprogesterone	Acetone	NADP
20β-HSD	17α-Hydroxyprogesterone	Acetone	NAD

NAD: Nicotinamide adenine dinucleotide, NADP: nicotinamide adenine dinucleotide phosphate.

oocyte complexes (COCs) cultured for 22 hrs with olomoucine is significantly smaller than control COCs cultured without olomoucine, and suggested that the synthesis of cAMP in cumulus cells and the transfer of cAMP from cumulus cells to oocytes are inhibited by the treatment of olomoucine (Takano and Niimura, 2004). Therefore, the reason for no changes in steroid metabolism and the size of lipid droplets in oocytes treated with olomoucine is thought to be the low cAMP level in their cytoplasm. However, the steroid metabolism and the size of lipid droplets in oocytes in which resumption of meiotic division was inhibited and the high cAMP level in the cytoplasm was maintained have not yet been determined.

On the other hand, 3-isobutyl-1-methylxanthine (IBMX) is known to be an inhibitor of cAMP phosphodiesterase, which metabolizes cAMP to 5'-AMP, and has the effect to maintain cAMP at a higher level in the cytoplasm (Shimada *et al*., 2002; Fan *et al*., 2002). It has been confirmed that maintaining the cAMP at a higher level by IBMX treatment results in the suppression of resumption of maturation division in mammalian oocytes (Magnusson and Hillensjö, 1977; Bilodeau *et al*., 1993; Tsafriri *et al*., 1996; Sun *et al*., 1999; Hegele-Hartung *et al*., 2001). In addition, when cAMP level in the cytoplasm was maintained at a higher level in oocytes treated with dibutyryl cAMP (dbcAMP), an analogue of cAMP, the rates of oocytes whose germinal vesicles (GVs) had broken down significantly decreased (Petr *et al*., 1991; Mattioli *et al*., 1994; Funahashi *et al*., 1997).

In the present investigation, the activities of HSDs and the number of Sudanophilic lipid droplets were histochemically observed in porcine oocytes treated with IBMX or dbcAMP, which suppresses the nuclear maturation with the mechanism different from olomoucine, in order to clarify the roles of cAMP in the metabolism of steroids and the number of lipid droplets, and the relationship between nuclear maturation and the metabolism of steroids and the number of lipid droplets in the cytoplasm.

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 ℃ . The ovaries were washed in 0.9 % NaCl solution containing 200 i.u./ml potassium penicillin G. 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) (Dulbecco and Vogt, 1954) 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) (Yoshida *et al*., 1990). These COCs were cultured for 22 hrs at 39 ℃ in the culture medium containing 500μM IBMX (Sigma Chemical Co. MO, USA) or 2.0 mM dbcAMP (Sigma Chemical Co.). IBMX was previously dissolved in dimethyl sulfoxide (DMSO) and then diluted with the culture medium up to 500μM. The concentration of DMSO in the culture medium was adjusted to 0.1 % (v/v), and COCs cultured for 22 hrs in the medium containing DMSO at 0.1 % or in the medium with no dbcAMP were used as controls.

After culture, cumulus cells were dispersed from the oocytes by pipetting in PBS containing 0.1 % hyaluronidase (Sigma Chemical Co.).

Observation of nuclear maturation

In order to investigate nuclei, the denuded oocytes were fixed in 25 % (v/v) acetic acid in ethanol for 48 hrs at room temperature. The fixed oocytes were stained with 1.0 % aceto-orcein and examined for evidence of nuclear maturation under a light microscope.

To determine the viability of oocytes treated with IBMX or dbcAMP, progression of nuclear maturation was also observed in those further cultured for 22 hrs in the medium without IBMX and dbcAMP.

Observation of HSD activities and Sudanophilic lipids

In order to detect the activities of HSDs shown in **Table 1**, the method used by Niimura and Ishida (1976) was employed: the denuded oocytes were placed at 37 ℃ in a solution containing 1.8 mg substrate (Sigma Chemical Co.) which had been dissolved in 0.5 ml acetone or dimethylformamide, 4.0 mg cofactor (Sigma Chemical Co.), 2.0 mg nitroblue tetrazolium salt (Sigma Chemical Co.), and 10.0 ml 0.1 M phosphate buffer solution (pH 7.5). Oocytes

Table 2. Nuclei of porcine oocytes cultured with IBMX or dbcAMP

Treatments	No. of oocytes examined	Germinal vesicle	≦	Diakinesis	No. and (%) of oocytes at the stages of		
					Diakinesis	Metaphase I	Metaphase II
None	90	3 (3)[b]		87 (97)[a]	14 (16)	63 (72)	10 (12)
IBMX	74	72 (97)[a]		2 (3)[b]	2 (100)	0 (0)	0 (0)
None	46	10 (22)[b]		36 (78)[a]	14 (39)	22 (61)	0 (0)
dbcAMP	57	57 (100)[a]		0 (0)[b]	0 (0)	0 (0)	0 (0)

The oocytes were observed after 22 hrs of culture.
Values with different superscripts in the same column in each experimental lot are significantly different ($P<0.05$).

incubated in a solution containing the substrate solvent, acetone or dimethylformamide, but devoid of the substrates were observed as negative controls. The incubation time was exactly 150 min, because this was the period within which unspecified or endogenous dehydrogenase reactions never appeared in the control oocytes incubated in a substrate-free solution. The same procedures for the demonstration of HSDs were applied 3 times. Atretic oocytes were eliminated from the observation. As the histochemical reaction of HSD depends upon the reaction of $NADH_2$ dehydrogenase ($NADH_2$-DH) or $NADPH_2$ dehydrogenase ($NADPH_2$-DH) (Baillie *et al*., 1966), the demonstration of these enzymes was carried out according to the method of Barka and Anderson (1965). As negative controls, some oocytes were incubated in a substrate-free solution for 90 min at 37 ℃ . After incubation in the substrate solution, oocytes were washed in PBS, and were placed on glass slides to be photographed under a light microscope (OPTIPHOT-2, Nikon, Tokyo, Japan).

In order to demonstrate Sudanophilic lipids, the denuded oocytes were fixed in PBS containing 10 % formalin, and then stained with Sudan Ⅳ (McManus and Mowry, 1964). After staining, the oocytes were washed in PBS and placed on glass slides to be photographed under a light microscope. The size of Sudanophilic lipid droplets was classified into three groups using a micrometer under a light microscope as in previous our report (Niimura *et al*., 2002): small droplets less than 2.5 μm in diameter, medium ones 2.5-4.9 μm and large ones more than 5.0 μm. It was impossible to accurately count the number of small and medium lipid droplets because there were so many, the total was estimated on a 2-point scale: many (++) and few (+). The number of large lipid droplets was counted under the microscope. The same staining procedures were repeated 3 times, using 30 oocytes in each culture. Degenerated oocytes were eliminated from the observation.

Statistical analysis

The rates concerning nuclear maturation of cultured oocytes and the number of oocytes with HSD activities were statistically analyzed by Chi-square test. The number of large Sudanophilic lipid droplets was statistically analyzed by One-way analysis of variance (ANOVA).

RESULTS

Nuclear maturation

Nuclear maturation in porcine oocytes treated with IBMX or dbcAMP is shown in **Table 2**. Of oocytes cultured for 22 hrs in the IBMX-free medium and in the dbcAMP-free medium, 3 (3/90) and 22 % (10/46) were in the GV stage, respectively, and the remaining 97 and 78 % were in the diakinesis to metaphase Ⅱ (M Ⅱ) stages, mostly in the M Ⅰ stage (72 and 61 %). On the other hand, almost all nuclei of the oocytes treated with IBMX or dbcAMP were in the GV stage (97 and 100 %). The percentages of oocytes in the GV stage were significantly higher in both the treated oocytes than in control oocytes ($P<0.05$). Therefore, it is confirmed that treatments of IBMX and dbcAMP are able to inhibit the resumption of nuclear maturation in porcine oocytes.

When the oocytes treated with IBMX or dbcAMP were further cultured for 22 hrs in the medium without IBMX and dbcAMP, most nuclei were in the M Ⅱ stage (90 and 76 %), suggesting that the ability of maturation in both IBMX-treated and dbcAMP-treated oocytes was sustained.

Activities of HSDs

When porcine oocytes cultured for 22 hrs were immersed in a substrate solution, diformazan granules were found to be deposited in the cytoplasm, as in our previous report (Takano and Niimura, 2002). Since such granules were not observed in the oocytes immersed in a solution containing no substrate (negative control), the granules were confirmed to represent the activity of HSDs. Using the method of Barka and Anderson (1965) for the demonstration of $NADH_2$-DH and $NADPH_2$-DH, diformazan granules were deposited in the cytoplasm of every oocyte. These granules did not appear in the negative control oocyte.

The activities of Δ^5-3β-HSD (pregnenolone and 17α-hydroxypregnenolone), 17β-HSD (estradiol-17β), 20α-HSD (20α-hydroxyprogesterone) and 20β-HSD (17α-hydroxyprogesterone) in porcine oocytes treated with IBMX or dbcAMP are shown in **Table 3**.

The percentages of the treated oocytes showing the activities of such HSDs did not differ from those of control oocytes cultured in the medium without IBMX and dbcAMP.

Table 3. The activities of HSDs in porcine oocytes cultured with IBMX or dbcAMP

Treatments	Δ5-3β-HSD				17β-HSD		20α-HSD		20β-HSD	
	Pregnenolone 1)		17α-Hydroxy-pregnenolone 1)		Estradiol-17β 1)		20α-Hydroxy-progesterone 1)		17α-Hydroxy-progesterone 1)	
	+2)	−2)	+	−	+	−	+	−	+	−
None	9(10)*a	83(90)a	2(2)a	100(98)a	10(10)a	87(90)a	8(7)a	101(93)a	2(2)a	97(98)a
IBMX	7(12)a	51(88)a	2(3)a	61(97)a	9(15)a	53(85)a	3(5)a	61(95)a	0(0)a	67(100)a
None	0(0)*a	65(100)a	0(0)a	71(100)a	0(0)a	73(100)a	9(13)a	63(88)a	0(0)a	69(100)a
dbcAMP	2(3)a	78(98)a	0(0)a	63(100)a	4(5)a	70(95)a	4(5)a	72(95)a	2(3)a	75(97)a

The oocytes were observed after 22 hrs of culture.
1) Substrates for enzyme-histochemistry.
2) + Positive, − negative.
* The number of oocytes with percentages in parentheses.
Values with different superscripts in the same column in each experimental lot are significantly different ($P<0.05$).

Table 4. The number of Sudanophilic lipid droplets of different sizes in porcine oocytes cultured with IBMX or dbcAMP

Treatments	No. of oocytes examined	No. of lipid droplets		
		Small (<2.5μm)	Medium (2.5-4.9μm)	Large (≧5.0μm)
None	30	++	++	55±4.64a
IBMX	30	++	++	47±3.76a
None	30	++	++	62±4.64a
dbcAMP	30	++	++	51±3.87a

++ represents many.
Data show mean ± S.E.
Values with different superscripts in the same column in each experimental lot are significantly different ($P<0.05$).

Number of Sudanophilic lipids

Sudanophilic lipids were observed as reddish-orange droplets of different sizes in the cytoplasm when porcine oocytes cultured for 22 hrs were stained with Sudan Ⅳ, as in our previous report (Niimura *et al*., 2002).

As shown in **Table 4**, the oocytes treated with IBMX or dbcAMP had many small and medium Sudanophilic lipid droplets, and 47±3.76 and 51±3.87 large ones, respectively. The control oocytes had also many small and medium lipid droplets, and 55±4.64 and 62±4.64 large ones, respectively. The amount and number of lipid droplets of different sizes in the treated oocytes did not differ from those in control oocytes.

DISCUSSION

As previously mentioned, we have observed in porcine oocytes that the metabolic abilities of progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, 17α,20β-dihydroxyprogesterone and estradiol-17β, and the size of Sudanophilic lipid droplets decrease as the nuclear maturation progresses (Niimura *et al*., 2002; Takano and Niimura, 2002). We have also observed the metabolism of such steroids and the number of lipid droplets in porcine oocytes treated with olomoucine, in order to determine the relationship between nuclear maturation and changes in the steroid metabolism and the number of lipid droplets in the cytoplasm. The resumption of nuclear maturation was completely inhibited in olomoucine-treated oocytes, and the decrease in the steroid metabolism and the reduction in the size of lipid droplets were also inhibited in those treated with olomoucine (Niimura *et al*., 2002; Takano and Niimura, 2002). From these results, we suggested that these changes in the cytoplasm are associated with progression of nuclear maturation. However, whether olomoucine directly acts on the cytoplasm to inhibit such changes could not be determined. On the other hand, we have recently reported that the resumption of nuclear maturation is completely inhibited in porcine oocytes treated with IBMX or dbcAMP, whereas the movement of cortical granules to the cytoplasm immediately beneath the plasma membrane is not inhibited (Takano and Niimura, 2008). Furthermore, we have also observed that the amount of cAMP is significantly smaller in porcine COCs treated with olomoucine than in control COCs, and suggested that the synthesis of cAMP in cumulus cells is inhibited and the transfer of cAMP from cumulus cells to oocytes does not occur by the treatment of olomoucine

(Takano and Niimura, 2004). Therefore, the reason for no changes in steroid metabolism and the size of lipid droplets in oocytes treated with olomoucine is thought to be the low cAMP level in their cytoplasm. However, the steroid metabolism and the size of lipid droplets in oocytes in which resumption of meiotic division was inhibited and the high cAMP level in the cytoplasm was maintained have not yet been determined.

In the present investigation, we attempted to observe the activities of some HSDs and the number of Sudanophilic lipid droplets, using oocytes treated with IBMX or dbcAMP, which suppresses the nuclear maturation with the mechanism different from olomoucine, in order to clarify the roles of cAMP in the metabolism of steroids and the number of lipid droplets. As a result, the resumption of nuclear maturation did not occur in the treated oocytes, and the activities of HSDs and the number of lipid droplets in the treated oocytes did not differ from those of control oocytes. From these results, it was suggested that the changes in the steroid metabolism and the size of lipid droplets in the cytoplasm with oocyte maturation depend on the cAMP level in their cytoplasm rather than the progression of nuclear maturation, and that the reason for no changes in the cytoplasm of olomoucine-treated oocytes is the low cAMP level in their cytoplasm. Also, the higher cAMP level in the cytoplasm is thought to be related to not only inhibition of nuclear maturation but also progression of cytoplasmic maturation, and the cAMP level is considered to be the important factor for cytoplasmic maturation of oocytes. It is generally known that cAMP plays a role as a second messenger to hormone actions in the steroidogenesis of luteal cells (Marsh *et al*., 1966; Marsh, 1971; Rao, 1973; Herlitz *et al*., 1974) and in the lipolysis of fat cells (Brasaemle *et al*., 2000; Morimoto *et al*., 2001), respectively. Therefore, it was considered that higher cAMP level in the cytoplasm of treated oocytes is also involved in the steroidogenesis and the lipolysis in their cytoplasm.

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IBMX および dbcAMP で処置したブタ卵母細胞における核の成熟、ステロイド代謝および脂質小滴の数

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（平成20年８月３日受付）

要　約

我々は、ブタ卵母細胞において、核の成熟に伴って細胞質ではいくつかのステロイドの代謝低下と脂質小滴の小型化が起こることを以前報告した。また、核の成熟をオロモウシンで処置して阻止した卵母細胞では、ステロイド代謝の低下および脂質小滴の小型化はみられなかったことから、細胞質でのこれらの変化は核の成熟と密接に関係していることを推察して報告した。しかし、オロモウシンがステロイド代謝の低下および脂質小滴の小型化に直接関係しているのか否かは明らかにできなかった。そこで、オロモウシンとは異なった機構、すなわち細胞質の cAMP レベルを高く維持することによって核の成熟を抑制する作用のある IBMX と dbcAMP を用いて、ブタの卵母細胞を処置し、ステロイド代謝と脂質小滴の数を組織化学的に観察した。

IBMX あるいは dbcAMP で22時間処置した卵母細胞において、核は97および100％で卵核胞期にあり、これらの処置により成熟分裂の再開が抑制されていることが確認された。一方、IBMX あるいは dbcAMP で22時間処置した卵母細胞において、Δ^5-3β-HSD 活性（基質として pregnenolone と17α-hydroxypregnenolone を使用）、17β-HSD 活性（estradiol-17β）、20α-HSD 活性（20α-hydroxyprogesterone）および20β-HSD 活性（17β-hydroxyprogesterone）を有するものの割合、ならびに各種大きさの脂質小滴の数は、どちらも対照の卵母細胞におけるそれらと相違なかった。

以上の結果から、細胞質におけるステロイド代謝の低下および脂質小滴の小型化は、核の成熟の進行よりも、細胞質の cAMP の量に依存して起こることが推察された。

新大農研報, 61(1):27-33, 2008

キーワード：ブタ卵母細胞、cAMP、ステロイド代謝、脂質小滴、組織化学

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