Changes of Steroid Metabolism in Mouse Oocytes during Meiotic Maturation

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ABSTRACT: The activities of hydroxysteroid dehydrogenases (HSDs) were histochemically demonstrated in mouse oocytes in the process of maturation in vivo and in vitro, and the changes in steroid metabolism during meiotic maturation were examined. In mouse oocytes soon after collection, the activities of Δ^5-3β-HSD, 17β-HSD and 20β-HSD were observed in 87 to 97% of those, while the activity of 20α-HSD was not. The percentages of oocytes showing the activities of Δ^5-3β-HSD (using DHA, pregnenolone and 17α-hydroxypregnenolone as the substrates), 17β-HSD (estradiol-17β and testosterone) and 20β-HSD (17α-hydroxyprogesterone and 20β-hydroxyprogesterone) did not change during maturation in vivo and in vitro. The oocytes with the activity of 20α-HSD (20α-hydroxyprogesterone) appeared 4 hrs after the hCG injection or after culturing for 4 hrs and the rates of those reached 92 and 100%, respectively, 14 hrs after the hCG injection or after culturing for 14 hrs. In the oocytes cultured for 8 hrs with olomoucine, an inhibitor of cyclin-dependent kinase, nuclei were almost in the germinal vesicle stage, and the activity of 20α-HSD (20α-hydroxyprogesterone) was observed in 84% of the treated oocytes. On the other hand, 81% of control oocytes also showed such the HSD activity, showing no difference from the rate of the treated oocytes.

From the present findings, it was suggested that the metabolic abilities of progesterone, 17α-hydroxyprogesterone, 17α,20β-dihydroxyprogesterone, 20β-hydroxyprogesterone, estradiol-17β and androgen are constantly present in mouse oocytes in the process of maturation in vivo and in vitro. And it was also suggested that the metabolic ability of 20α-hydroxyprogesterone in mouse oocytes varies with maturation, while the change in the metabolic ability of such steroid is not related to nuclear maturation.

Key words: mouse oocyte, meiotic maturation, hydroxysteroid dehydrogenase, steroid metabolism, histochemistry

INTRODUCTION

Recently, it has been shown that C-29 sterols, intermediates in the cholesterol biosynthetic pathway, are involved in oocyte maturation. Namely, meiosis-activating sterols (MASs) that induce the resumption of nuclear maturation have been determined as 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol (FF-MAS) and 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol (T-MAS) in mammalian oocytes of several species[1-3].

It has also been reported that steroid metabolism in the cytoplasm alters with nuclear maturation of oocytes. Namely, it has been biochemically confirmed that the activity of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) with pregnenolone as the substrate, which is involved in progesterone metabolism, increases in rat oocytes after germinal vesicle breakdown (GVBD), and that this activity reaches a peak at the time of ovulation, indicating that nuclear maturation in rat oocytes is associated with progesterone metabolism[4]. We have also reported that the activities of Δ^5-3β-HSD (using 17α-hydroxypregnenolone as the substrate) and 20β-HSD (17α-hydroxyprogesterone) were observed in almost all oocytes collected from antral follicles of mice, rabbits, pigs and cattle, and that they disappeared from the oocytes of these animals as nuclear maturation progressed, except for the mouse[5]. From these results, it was inferred that the nuclear maturation of rabbit, porcine and bovine oocytes is closely related to 17α-hydroxyprogesterone metabolism, and 17α,20β-dihydroxyprogesterone metabolized from 17α-hydroxy-

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progesterone may possibly serve as a substance inducing the maturation of oocytes of these animals[5]. Changes in the metabolic abilities of steroids in mammalian oocytes with maturation have been investigated only in rats[4], mice[5], rabbits[5], pigs[5] and cattle[5], as mentioned above, though sufficient numbers of different kinds of HSDs were not demonstrated in those studies.

In the present study, the activities of various kinds of HSDs were histochemically demonstrated in mouse oocytes in the process of maturation in vivo and in vitro using as many substrates as possible, in order to investigate the changes in the metabolic abilities of steroids with oocyte maturation. As for HSD which showed changes in activity according to maturation, we also attempted to demonstrate the activity in oocytes treated with olomoucine[6], an inhibitor of cyclin-dependent kinase such as maturation-promoting factor, in order to clarify the relationship between nuclear maturation and change in the metabolic ability of steroid in the cytoplasm.

MATERIALS AND METHODS

Animals

Three hundred and eighty female mature mice of ICR strain were used in the present study. They were housed in autoclaved metal cages and were given a standard chow (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* in an air-conditioned room (24℃), under controlled-lighting conditions (14L/10D). These mice were intraperitoneally injected with 5 i.u. of PMSG (Serotropin®, Teikoku Hormone Manufacturing Co. Ltd., Tokyo, Japan).

Collection and culture of oocytes

In order to observe oocytes in the process of maturation in vitro, immature oocytes covered with cumulus cells (COCs) were collected from antral follicles 48 hrs after the PMSG injection and cultured in TYH medium[7] containing 5% fetal bovine serum (FCS, Gibco BRL, NY, USA) and 10 i.u./ml PMSG at 37℃ in a CO_2 incubator (5% CO_2 in air). On the other hand, PMSG-injected females were further intraperitoneally injected with 5 i.u. of hCG (Gonatropin®, Teikoku Hormone Manufacturing Co. Ltd.) 48 hrs after the PMSG injection to obtain oocytes in the process of maturation in vivo. COCs were collected from antral follicles or oviducts 4, 8 and 14 hrs after the hCG injection, respectively.

Observation of HSD activities

At 0, 4, 8 and 14 hrs after the hCG injection and at 4, 8 and 14 hrs after maturation culture, COCs were immersed in phosphate buffered saline (PBS, pH 7.4)[8] containing 0.1% hyaluronidase (Sigma-Aldrich, NJ, USA) to disperse their cumulus cells. In order to detect the activities of HSDs shown in **Table 1**, the method used by NIIMURA and ISHIDA[9] was employed: denuded oocytes were placed at 37℃ in a solution containing 1.8 mg substrate (Sigma-Aldrich) which had been dissolved in 0.5 ml acetone or dimethylformamide, 4.0 mg cofactor (Sigma-Aldrich), 2.0 mg nitroblue tetrazolium salt (Sigma-Aldrich), and 10.0 ml 0.1 M phosphate buffer solution (pH 7.5). Oocytes 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 60 to 120 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 to oocytes from each period after the hCG injection or maturation culture. Atretic oocytes were eliminated from the observation. As the histochemical reaction of HSDs depends upon the reaction of $NADH_2$ dehydrogenase ($NADH_2$-DH) or $NADPH_2$ dehydrogenase ($NADPH_2$-DH)[10], the demonstration of these enzymes was carried out according to the BARKA and ANDERSON method[11]. As negative controls, some oocytes were incubated in a substrate-

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

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

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

free solution for 60 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).

Observation of nuclear maturation

In order to investigate nuclei, oocytes cultured for various periods and those collected from antral follicles and oviducts were fixed in 25% (v/v) acetic acid in ethanol for 24 hrs at 4℃. The fixed oocytes were stained with 1.0% aceto-orcein and examined for evidence of nuclear maturation under a light microscope.

Observation of HSD activities and nuclear maturation in olomoucine-treated oocytes

To observe the activities of HSDs in oocytes in which resumption of meiotic division was inhibited, COCs collected from antral follicles 48 hrs after the PMSG injection were cultured for 8 hrs at 37℃ in TYH medium[7] containing 400 μM olomoucine (Sigma-Aldrich), which was previously dissolved in dimethyl sulfoxide (DMSO) and then diluted with the culture medium up to 400 μM. The concentration of DMSO in the culture medium was adjusted to 0.37% (v/v), and oocytes cultured in the medium containing DMSO at 0.37% for 8 hrs were used as vehicle controls. After culture, histochemical demonstration of the activity of 20α-HSD (20α-hydroxyprogesterone) was performed, as described above and then the presence of HSD activities was observed under a light microscope. To ensure the inhibition of resumption of meiotic division by olomoucine treatment, oocytes cultured for 8 hrs with olomoucine were fixed in 25% (v/v) acetic acid in ethanol, stained with 1.0% aceto-orcein and then examined under a light microscope. To determine the viability of the olomoucine-treated oocytes, progression of nuclear maturation was also observed in those further cultured for 14 hrs in the medium containing no olomoucine.

Statistical analysis

The rates concerning nuclear maturation and the number of oocytes with HSD activities were statistically analyzed by Chi-square test.

Table 2. Nuclear maturation of mouse oocytes

Maturation	Hrs after hCG injection or culture	No. of oocytes examined	No. and (%) of oocytes at the stage of					
			Germinal vesicle	Diakinesis	Metaphase I	Anaphase I	Telophase I	Metaphase II
in vivo	0	52	48(83)*	9(17)	0(0)	0(0)	0(0)	0(0)
	4	35	3(9)	21(60)	11(31)	0(0)	0(0)	0(0)
	8	41	0(0)	0(0)	31(76)	3(7)	6(15)	1(2)
	14	45	0(0)	0(0)	8(18)	2(4)	1(2)	34(76)
in vitro	0	52	48(83)	9(17)	0(0)	0(0)	0(0)	0(0)
	4	33	0(0)	11(33)	21(64)	1(3)	0(0)	0(0)
	8	40	0(0)	0(0)	29(73)	8(20)	3(8)	0(0)
	14	30	0(0)	0(0)	1(3)	2(7)	2(7)	25(83)

*The number of oocytes with percentages in parentheses.

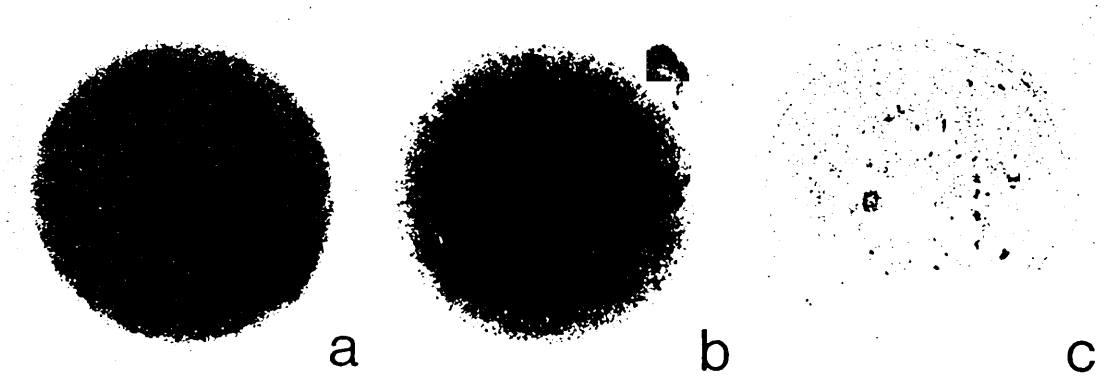

Fig. 1. Diformazan granules showing the presence of the activity of 17β-HSD with estradiol-17β as the substrate (a) and Δ^5-3β-HSD with DHA as the substrate (b) are deposited in the cytoplasm of oocytes, but not in the cytoplasm of a negative control oocyte, which was incubated in a substrate-free solution (c). All the pictures were taken at a magnification of ×350.
(a and c) Oocytes soon after collection, (b) an oocyte 14 hrs after culture.

RESULTS

Nuclear maturation

Immediately after collection, nuclei of oocytes were in the germinal vesicle (GV) and diakinesis stages, mostly in the GV stage (83%). The percentage of oocytes at the GV stage decreased over time of maturation in vivo and in vitro and reached 0% after culturing for 4 hrs or 8 hrs after the hCG injection. Nuclei of oocytes cultured for 8 hrs or 8 hrs after the hCG injection were almost in the metaphase I (MI) stage, 73 and 76%, respectively. Of oocytes cultured for 14 hrs or 14 hrs after the hCG injection , 83 and 76%, respectively, were in the MII stage (**Table 2**).

Activities of HSDs

When mouse oocytes in the process of maturation in vivo and in vitro were immersed in a substrate solution, diformazan granules were found to be deposited in the cytoplasm (**Fig. 1a** and **b**). Since such

Table 3. Activities of HSDs in mouse oocytes during meiotic maturation in vivo

Hours after hCG injection	Δ^5-3β-HSD						17β-HSD				20α-HSD		20β-HSD			
	DHA[1]		Pregnenolone[1]		17α-Hydroxy-pregnenolone[1]		Estradiol-17β[1]		Testosterone[1]		20α-Hydroxy-progesterone[1]		17α-Hydroxy-progesterone[1]		20β-Hydroxy-progesterone[1]	
	+[2]	−[2]	+	−	+	−	+	−	+	−	+	−	+	−	+	−
0	40(91)*	4(9)	37(95)	2(5)	58(89)	7(11)	39(87)	6(13)	41(89)	5(11)	0(0)	57(100)	36(92)	3(8)	38(97)	1(3)
4	30(97)	1(3)	31(97)	1(3)	63(93)	5(7)	25(96)	1(4)	31(100)	0(0)	52(67)	26(33)	29(94)	2(7)	28(90)	3(10)
8	37(93)	3(8)	38(100)	0(0)	46(92)	4(8)	36(86)	6(14)	40(89)	5(11)	67(91)	7(10)	32(89)	4(11)	37(90)	4(10)
14	62(98)	1(2)	61(97)	2(3)	53(92)	5(9)	72(92)	6(8)	65(100)	0(0)	96(92)	8(8)	75(95)	4(5)	58(88)	8(12)

[1] Substrates for enzyme-histochemistry.
[2] +Positive, −negative.
*The number of oocytes with percentages in parentheses.

Table 4. Activities of HSDs in mouse oocytes during meiotic maturation in vitro

Hours of culture	Δ^5-3β-HSD						17β-HSD				20α-HSD		20β-HSD			
	DHA[1]		Pregnenolone[1]		17α-Hydroxy-pregnenolone[1]		Estradiol-17β[1]		Testosterone[1]		20α-Hydroxy-progesterone[1]		17α-Hydroxy-progesterone[1]		20β-Hydroxy-progesterone[1]	
	+[2]	−[2]	+	−	+	−	+	−	+	−	+	−	+	−	+	−
0	40(91)*	4(9)	37(95)	2(5)	58(89)	7(11)	39(87)	6(13)	41(89)	5(11)	0(0)	57(100)	36(92)	3(8)	38(97)	1(3)
4	28(97)	1(3)	34(81)	8(19)	31(97)	1(3)	30(100)	0(0)	28(93)	2(7)	40(67)	20(33)	40(83)	8(17)	28(93)	2(7)
8	30(100)	0(0)	29(88)	4(12)	31(100)	0(0)	28(93)	2(7)	31(100)	0(0)	33(92)	3(8)	28(100)	0(0)	28(100)	0(0)
14	31(100)	0(0)	33(100)	0(0)	36(97)	1(3)	30(97)	1(3)	27(87)	4(13)	32(100)	0(0)	33(94)	2(6)	31(100)	0(0)

[1] Substrates for enzyme-histochemistry.
[2] +Positive, −negative.
*The number of oocytes with percentages in parentheses.

granules were not observed in the oocytes immersed in a solution containing no substrate (negative control; **Fig. 1c**), the granules were considered to represent the activity of HSDs. Using the method of BARKA and ANDERSON[11] for the demonstration of $NADH_2$-DH and $NADPH_2$-DH, deposited diformazan granules were found in the cytoplasm of every oocyte from each period after the hCG injection or maturation culture. These granules did not appear in the negative control oocytes.

The activities of various HSDs in mouse oocytes in the process of maturation in vivo and in vitro are shown in **Tables 3** and **4**. Of oocytes immediately after collection, 87 to 97% showed the activity of Δ^5-3β-HSD, 17β-HSD and 20β-HSD with seven substrates. The rates of oocytes with the activities of Δ^5-3β-HSD (DHA, pregnenolone and 17α-hydroxypregnenolone), 17β-HSD (estradiol-17β and testosterone) and 20β-HSD (17α-hydroxyprogesterone and 20β-hydroxyprogesterone) did not change during maturation in vivo and in vitro. On the other hand, oocytes with 20α-HSD activity were not found immediately after collection, while they appeared 4 hrs after the hCG injection or after culturing for 4 hrs and the rates of those reached 92 and 100%, respectively, 14 hrs after the hCG injection or after culturing for 14 hrs.

HSD activity and nuclear maturation in olomoucine-treated oocytes

The percentages of the olomoucine-treated oocytes showing the activity of 20α-HSD (20α-hydroxyprogesterone) were 84%. The percentages did not differ from 81% of the control oocytes. Most nuclei of the olomoucine-treated oocytes were in the GV stage (97%), while those of the control oocytes were in the diakinesis to telophase I stages, mostly in the MI stage (83%, 25/30).

When olomoucine-treated oocytes were further cultured in the olomoucine-free medium for 14 hrs, most nuclei of those were in the MII stage (90%, 28/31), suggesting that the ability to mature in the

olomoucine-treated oocytes was sustained.

DISCUSSION

In the present investigation, the activities of Δ^5-3β-HSD (DHA, pregnenolone and 17α-hydroxypregnenolone), 17β-HSD (estradiol-17β and testosterone) and 20β-HSD (17α-hydroxyprogesterone and 20β-hydroxyprogesterone) were almost always demonstrated in mouse oocytes during meiotic maturation in vivo and in vitro. Furthermore, the activity of 20α-HSD (20α-hydroxyprogesterone) appeared in the oocytes 4 hrs after the hCG injection or after culturing for 4 hrs and increased in number as the time of maturation was prolonged. Because nuclear maturation was found to progress in the oocytes observed in the present study, as in previous reports[12,13],nuclear maturation was considered to progress normally. From these results, it is suggested that the metabolic abilities of androgen, progesterone, 17α-hydroxyprogesterone, 17α,20β-dihydroxyprogesterone, 20β-hydroxyprogesterone and estradiol-17β remained unchanged, while the metabolic ability of 20α-hydroxyprogesterone appeared, regardless of maturation in vivo or in vitro.

We also attempted to demonstrate the HSD activity which had changed with maturation using olomoucine-treated oocytes in order to determine the relationship between nuclear maturation and change in metabolic ability of steroid in the cytoplasm. The resumption of nuclear maturation was almost completely inhibited in the olomoucine-treated oocytes, while the activity of 20α-HSD (20α-hydroxyprogesterone) was comparable between the olomoucine-treated oocytes and the control oocytes. Therefore, we consider that nuclear maturation in mouse oocytes is not associated with the metabolic ability of 20α-hydroxyprogesterone in the cytoplasm.

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マウス卵母細胞の成熟に伴うステロイド代謝の変化

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摘　　要

体内および体外で成熟過程にあるマウス卵母細胞について、各種ヒドロキシステロイド脱水素酵素（HSD）の活性を組織化学的に検出し、卵母細胞の成熟に伴うステロイド代謝能の変化を検討した。採取直後の卵母細胞において、87ないし97%でΔ^5-3β-HSD、17β-HSDおよび20β-HSDの活性が検出されたが、20α-HSDの活性は検出されなかった。一方、Δ^5-3β-HSD(基質としてDHA、pregnenoloneおよび17α-hydroxypregnenoloneを使用)、17β-HSD (estradiol-17β と testosterone) および20β-HSD (17α-hydroxyprogesterone と20β-hydroxyprogesterone) の活性を示す卵母細胞の割合は、体内および体外での成熟の経過に伴う変化を示さなかったが、成熟に伴って、20α-HSD (20α-hydroxyprogesterone) の活性を示す卵母細胞は出現し、その割合は高くなった。また、8時間オロモウシン処置した卵母細胞において、核はほとんどで卵核胞期にあるとともに、20α-HSD (20α-hydroxyprogesterone) の活性を示すものの割合は84%であり、オロモウシン処置していない対照の卵母細胞の81%と相違なかった。

以上の結果から、マウス卵母細胞のprogesterone、17α-hydroxyprogesterone、17α,20β-dihydroxyprogesterone、20β-hydroxyprogesterone、estradiol-17βおよびandrogenの代謝能は、体内および体外での成熟に関わらず変化しないことが示唆された。また、マウス卵母細胞では、成熟に伴って20α-hydroxyprogesteroneの代謝能は出現して高まるが、この代謝能の変化は、核の成熟とは関係ないことが推察された。

キーワード：マウス卵母細胞、成熟分裂、ヒドロキシステロイド脱水素酵素、ステロイド代謝、組織化学

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