Inhibition of Polar Body Formation in Mouse Denuded Oocytes Cultured *in Vitro* by Protein Synthesis Inhibitors

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Denuded oocytes obtained from the ovaries of ddY mice underwent spontaneous germinal vesicle breakdown (GVBD) and polar body formation (PBF) by cultivation for 3 and 16 h, successively. To study the significance of protein synthesis in oocytes regarding their maturation, denuded oocytes were cultured in the presence of reversible (cycloheximide and puromycin) and irreversible (ricin) protein synthesis inhibitors. When denuded oocytes were cultivated in the continual presence of any inhibitor, only PBF but not GVBD was found to be inhibited. These inhibitions were dose dependent, ranging from 10^{-9} to 10^{-6} M, and were relative to the protein synthesis inhibitions by them. The PBF inhibition was relieved by withdrawing cycloheximide for first 2, 3 and 4 h in the course of the culture. Further, the PBF inhibition occurred in the presence of ricin for only the first 2 or 3 h in the course of the culture. These results suggested that proteins synthesized during 2 to 4 h in the course of culture may play important roles in PBF.

Key words oocyte maturation; polar body formation; germinal vesicle breakdown; ricin; cycloheximide

In mammals, oocytes in primary follicles arrest their meiotic progression at the dictyate stage (late prophase of the first meiotic division).¹⁾ Fully grown oocytes reinitiate meiosis in response to hormonal signals, such as pituitary gonadotropins and ovarian estrogen, at or near the time of ovulation *in vivo*.²⁾ The meiotic progression of oocytes is called the maturation of oocytes, and morphologically germinal vesicle breakdown (GVBD) and polar body formation (PBF) of oocytes are observed during maturation. Upon releasing the oocytes from the follicles *in vitro*, the meiotic progression resumes, leading to a spontaneous GVBD followed by PBF of the oocytes in culture.^{3–5)}

Methods for maintaining the culture of oocytes *in vitro* became available recently, and studies on regulatory mechanisms of oocyte maturation have been stimulated. A number of laboratories have demonstrated that some protein factors synthesized in oocytes are necessary for their spontaneous maturation; *i.e.*, in ungulates, GVBD of oocytes from bovine,^{6,7)} pig^{8,9)} and sheep¹⁰⁾ are reversibly inhibited by protein synthesis inhibitors such as cycloheximide and puromycin. In rats and mice, however, these protein synthesis inhibitors do not prevent GVBD, but block PBF^{11–15)} in the ordinary culture of oocytes. Using dbcAMP-treated oocytes, Ekholm and Magnusson¹⁵⁾ and Downs¹⁶⁾ have shown that in rodents the oocytes are not sensitive to protein synthesis inhibitors, because the oocytes become committed to undergoing GVBD before the proteins involved are turned over. Thus, the significance of the protein synthesis in oocytes for their maturation has been established; however, the specific period of protein synthesis in the course of culture, particularly in correlation with PBF, remains unknown.

In the present study, using reversible (cycloheximide and puromycin) and irreversible (ricin) protein synthesis inhibitors, we have confirmed that *de novo* protein synthesis is prerequisite to PBF but not to the GVBD of mouse denuded oocytes in ordinary culture. Furthermore, we attempted to determine the period of protein synthesis, which is correlated to PBF, in the course of the culture.

MATERIALS AND METHODS

Materials Ricin toxin was purified from Chinese castor beans according to the method of Hara *et al.*¹⁷⁾ [³⁵S]Labelling mix ([³⁵S]Met/Cys, >37 TBq/mmol) was obtained from Amersham. All other chemicals were of reagent grade and obtained from commercial sources.

***In Vitro* Culture of Mice Denuded Oocytes** The *in vitro* culture of denuded oocytes from prepubertal mice (ddY strain) was carried out by the method previously reported.¹⁸⁾ Briefly, the oocytes (15–30) were cultured in a drop of 0.1 ml of the culture medium¹⁹⁾ [modified Krebs–Ringer buffer solution (pH 7.2) supplemented with 0.25 mM pyruvate and 1 mg/ml bovine serum albumin] under paraffin oil. The oocytes were incubated for 20 h at 37 °C in a 5% CO₂ incubator. In the course of incubation, the oocytes were scored for GVBD and PBF by microscopic examination. Percent GVBD (% GVBD) was expressed as the percent of oocytes that underwent GVBD *vs.* the total oocytes analyzed. Percent PBF (% PBF) was expressed as the percent of oocytes that underwent PBF *vs.* oocytes which underwent GVBD scored at 3 h incubation.

Measurement of Protein Synthesis in Denuded Oocytes The denuded oocytes (10 oocytes for one group in most cases) were cultured in 50 μ l of medium containing [³⁵S]-Met/Cys (10.6 MBq/ml) in the absence or presence of either ricin, cycloheximide or puromycin. After incubation, oocytes were washed with fresh medium and were then transferred into 90 μ l of medium. The medium containing oocytes was mixed with 100 μ l of 20% trichloroacetic acid (TCA) solution after the addition of 10 μ l of 20% bovine serum albumin solution as a carrier protein for precipitation. The resulting precipitate was collected on a GF/C filter (Whatman) preswollen with 10% TCA and then washed with 10% TCA and ethanol, after which the radioactivity was measured.

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RESULTS

Maturation of Mice Denuded Oocytes *in Vitro* Mice denuded oocytes were cultured for 20 h under the conditions described in Materials and Methods, and the % GVBD and % PBF were examined at the periods indicated. Figure 1A shows that GVBD was observed in $84.5 \pm 0.7\%$ of oocytes examined after cultivation for 3 h. After 13 h more incubation, $76.2 \pm 1.4\%$ of oocytes that underwent GVBD were processed to PBF (Fig. 1B).

Effect of Protein Synthesis Inhibitors on Oocyte Protein Synthesis and Its Maturation *in Vitro* Figure 2 shows the effects of protein synthesis inhibitors on % GVBD and % PBF. When denuded oocytes were cultured in the continual presence of an inhibitor, PBF after 16 h culture was inhibited in a dose-dependent manner. Complete PBF inhibition was observed at the concentrations of 10^{-5} M for cycloheximide and 10^{-6} M for ricin and puromycin, respectively. However, GVBD after 3 h culture was not affected by any of the inhibitors. During cultivation with an inhibitor, no morphological abnormality of oocytes was observed. Protein syntheses in oocytes were also measured in the presence or absence of ricin and cycloheximide (data not shown). In the absence of inhibitors, protein synthesis in oocytes was increased with the time of cultivation and reached a plateau after 10 h cultivation. In the presence of inhibitors, however, protein synthesis in oocytes was decreased dose-dependently. The dose-dependent inhibition of protein synthesis by ricin and cycloheximide was similar to the inhibition of PBF, indicating that PBF inhibition by these inhibitors must be directly correlated to the inhibition of protein synthesis.

Effect of Protein Synthesis Inhibitor Treatments and Their Withdrawal on PBF of Mouse Oocytes Cultured *in Vitro* To examine the specific period of protein synthesis involved in PBF, oocytes were cultured in the presence of protein synthesis inhibitors with changes in the periods of their presence (Figs. 3, 4). As mentioned above, PBF was inhibited in the presence of cycloheximide or ricin, but this inhibition was relieved by withdrawal cycloheximide for first 2, 3 and 4 h of cultivation (B, C, D in Fig. 3). Similarly, PBF inhibition of ricin was also relieved by withdrawing for first 2 and 3 h of cultivation (D, E in Fig. 4). Furthermore, PBF was inhibited dose-dependently in the presence of ricin, an irreversible inhibitor of protein synthesis, for only the first 2 and 3 h of cultivation (C, B in Fig. 4). These results suggest that proteins synthesized during 2–4 h of oocyte cultivation might be related to the PBF of oocytes. On the other hand, the withdrawal of cycloheximide after 3 or 4 h of cultivation (presence of cycloheximide for first 3 or 4 h of cultivation) showed no effect on PBF inhibition, only a delay of PBF (E, F in Fig. 3). This may be caused by the reversibility of the effect of cycloheximide on protein synthesis inhibition.

DISCUSSION

De novo protein synthesis is essential for the maturation of most mammalian oocytes. Continuous inhibition of protein synthesis in the mouse oocytes in culture by cycloheximide or puromycin involves blocking the meiotic

Fig. 1. GVBD and PBF of Denuded Mouse Oocytes *in Vitro*

Cumulus-free oocytes were cultured in the medium and the percent GVBD (A) and PBF (B) that occurred spontaneously were examined at the indicated periods. Each point is the mean of several experiments and the mean of a number of individual experiments performed using 15–30 oocytes. Parentheses indicate the total number of oocytes used to determine each point.

progression from GVBD to PBF.^{11–15}) This was confirmed by the present study. However, the specific period of protein synthesis in the course of culture and characteristics of synthesized proteins correlated with PBF remained unknown.

The blocking effect of cycloheximide was fully reversible; *i.e.*, when the drug was removed from the culture medium of oocytes, protein synthesis in oocytes resumed. Accordingly, PBF inhibition caused by stopping the protein synthesis in oocytes was released by the removal of the drug from the medium, thus cycloheximide merely delayed the time of PBF. It may be impossible to specify the period of protein synthesis correlated with PBF using only the reversible inhibitor. Therefore, to determine the period of protein synthesis correlated with PBF, we used ricin as an irreversible protein synthesis inhibitor in addition to reversible inhibitor. Ricin is a protein toxin containing two polypeptide chains, A and B, that are covalently linked by a disulfide bond.²⁰) The A chain is an *N*-glycosidase that removes a single adenine residue from eukaryotic 28S RNA, which inactivates 60S ribosomal subunits and arrests protein synthesis.²¹)

Reversible and irreversible effects of cycloheximide and ricin on the inhibition of both protein synthesis and PBF

Fig. 2. Effect of Protein Synthesis Inhibitors on Mouse Oocyte Maturation *in Vitro*

Oocytes were cultured in the presence of one of the following three protein inhibitors: A, cycloheximide; B, puromycin; C, ricin. The percent GVBD (●) was examined after 3h culture and the percent PBF (○) was examined after 16h culture. Data are expressed as described in the legend to Fig. 1.

Fig. 3. Effect of Cycloheximide Treatment and Its Withdrawal on PBF of Mouse Oocytes Cultured *in Vitro*

Oocytes were cultured with the indicated dose of cycloheximide under the conditions described in A–F in the lower figure. After 16 to 19h cultivation, the percent PBF was examined.

Fig. 4. Effect of Ricin Treatment and Its Withdrawal on PBF of Mouse Oocytes Cultured *in Vitro*

Oocytes were cultured with the indicated dose of ricin under the conditions described in A–E in the lower figure. After 16 to 19h cultivation, the percent PBF was examined.

Table 1. Summary of the Effects of Protein Synthesis Inhibitors on Oocyte Maturation *in Vitro*

	Cultivation time (h)	% PBF ^{a)}	Protein ^{a)} synthesis (%)
Ricin 10 ⁻⁶ M	0		
	3		
	16		
	19	0	11.6
CH 10 ⁻⁵ M	4		
	5	87.5	96.6
	6	28.6	19.3

■: Presence of protein synthesis inhibitors. a) Determined after 16 or 19 h cultivation.

are summarized in Table 1. Even if cycloheximide was present for the first 3 h in the course of oocyte culture, neither PBF nor protein synthesis was inhibited after a total of 19 h of culture, indicating that the effect of cycloheximide was completely reversible. On the other hand, the effect of ricin was irreversible, since the first 3 h presence of ricin in the culture inhibited both PBF and protein synthesis of oocytes. In addition, when cycloheximide was present continually in the culture for 3–16 h in which protein synthesis in oocytes was decreased to 19.3% of control, PBF was also decreased to 28.6%. By contrast, under the same experimental conditions, ricin inhibited protein synthesis in oocytes but not PBF. This is probably caused by a lag time in the effect of ricin, since cycloheximide inhibited protein synthesis immediately, but ricin showed 1 and 3 h lag periods, at the concentrations of 10⁻⁶ and 10⁻⁸ M, respectively (data not shown). This may be due to the difference in incorporation into oocytes of cycloheximide (passive incorporation) and ricin (receptor-mediated endocytosis). Taking into account the 1 h lag time for the expression of the ricin effect (10⁻⁶ M), the protein synthesis in oocytes was not affected for the first 1 h after the addition of ricin to the medium. These

results indicate that PBF takes place when protein synthesis in oocytes is intact between 3 to 4 h in the course of culture. Considering that PBF was effectively inhibited by the addition of cycloheximide at 2 h after starting the culture (Fig. 3, B), synthesized proteins between 2 to 4 h in the course of culture must be correlated with PBF.

In conclusion, the importance of protein synthesis during 2–4 h in the course of mouse oocyte culture for meiotic maturation, from GVBD to PBF, is suggested. The determination of the proteins which are synthesized stage-specifically during 2–4 h in the course of the culture is now being studied and will be published elsewhere.

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