Changes in protein synthesis and phosphorylation patterns during bovine oocyte maturation *in vitro*

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Summary. Sequential protein synthesis and protein phosphorylation patterns were generated by radiolabelling bovine cumulus–oocyte complexes after various periods of culture with [³⁵S]methionine and [³²P]orthophosphate respectively. The radiolabelled oocytes were assessed for their nuclear status and used individually for gel electrophoresis. Marked changes in the protein synthesis patterns were observed exclusively after germinal vesicle breakdown (GVBD), whereas oocytes which remained in the germinal vesicle stage showed a consistent protein synthesis pattern. The changes were observed after 8 and 16 h of culture, shortly after GVBD and before first polar body extrusion. From 3 h of culture, dominant phosphoprotein bands with apparent molecular weights of 24 000 and two between 50 000 and 60 000 were observed. The latter bands displayed slight molecular weight changes, which were not closely time related. After GVBD, the phosphoprotein band with M_r 19 000 was no longer observed. This study demonstrates that specific changes in protein synthesis and protein phosphorylation are programmed during bovine oocyte maturation.

Keywords: cow; oocyte; maturation; protein synthesis; protein phosphorylation

Introduction

Mammalian oocytes are arrested at the diplotene stage of prophase I until just before ovulation. Resumption of meiosis can be induced by the transfer of oocytes from their ovarian follicles into a suitable culture medium (Pincus & Enzmann, 1935; Edward, 1965). In the ensuing period of culture, oocytes undergo nuclear progression from diplotene to metaphase II and the change is characterized by dissolution of the nuclear membrane (Germinal Vesicle Breakdown) and extrusion of the first polar body. However, mature oocytes have not only undergone nuclear, but also cytoplasmic, changes necessary for fertilization and subsequent embryonic development (Moor & Trounson, 1977). Structural rearrangements of organelles (Szöllösi, 1972; Cran *et al.*, 1980; Cran, 1985) and major changes in protein synthesis patterns (Golbus & Stein, 1976; Schultz & Wassarman, 1977; McGaughey & Van Blerkom, 1977; Warnes *et al.*, 1977) take place during cytoplasmic maturation and have been described for several mammalian species.

So far, only structural changes have been described (Fleming & Saacke, 1972; Kruip *et al.*, 1983; Hyttel *et al.*, 1986) for bovine oocytes. The present study examines protein synthesis and protein phosphorylation patterns during maturation of bovine oocytes *in vitro*.

Materials and Methods

Collection and culture of cumulus–oocyte complexes. Bovine ovaries, obtained at a local slaughterhouse, were rinsed and collected in phosphate-buffered saline (PBS; Dulbecco & Vogt, 1954) at 30°C and transported to the laboratory

within 1 h after slaughter. Antral follicles, between 4 and 8 mm in diameter, were aspirated and cumulus-oocyte complexes were recovered from the follicular fluid aspirates after sedimentation for 15 min. Subsequently, the cumulus-oocyte complexes were washed in PBS, supplemented with 4 mg bovine serum albumin/ml, 0.36 mM-pyruvate, 23.8 mM-lactate and 5.5 mM-glucose (Medium A), and classified under a stereomicroscope (magnification $\times 50$) according to De Loos *et al.* (1989). Only intact cumulus-oocyte complexes with a compact and multilayered cumulus investment were used in this study, since no differences in the protein synthesis patterns of these oocytes were observed in a previous study (Kastrop *et al.*, 1990). The selection procedure was carried out at 30°C and within 2–3 h after slaughter.

Cumulus-oocyte complexes were cultured in Medium 199 with Earle's salts (Flow Laboratories, Herts, UK), supplemented with 10% heat-treated fetal calf serum, 1 μ g bLH/ml, 2 μ g bFSH/ml and 1 μ g oestradiol-17 β /ml. Culture was carried out at 39°C in an humidified atmosphere of 5% CO₂ in air and all media were adjusted to pH 7.2 and 280 mosmol.

Radiolabelling of oocyte proteins. After various periods of culture, at least 20 cumulus-oocyte complexes per culture period were either radiolabelled with [³⁵S]methionine or [³²P]orthophosphate for 3 h at 39°C. To study protein synthesis, labelling was performed in Medium A, containing 1 mCi L-[³⁵S]methionine/ml (sp. act. > 1000 Ci/mmol; Amersham plc, Amersham, Bucks, UK). Phosphorylation of proteins was investigated by labelling cumulus-oocyte complexes with [³²P]orthophosphate (carrier free; Amersham plc) at a concentration of 0.5 mCi/ml, using bicarbonate-buffered saline, as described by Crosby *et al.* (1984). Labelled oocytes were denuded of cumulus cells by repeated pipetting and assessed for morphological appearance and nuclear status. Intact and non-degenerate oocytes were lysed individually by adding 20 μ l SDS-sample buffer (Laemmli, 1970), followed by heating at 100°C for 2 min. Samples were stored at –20°C and aliquants of 2 μ l were used for the determination of the incorporation of radiolabel into TCA-insoluble material.

Assessment of meiotic stage. After culture and radiolabelling, oocytes were denuded of cumulus cells by repeated pipetting and examined under a stereomicroscope for the presence of a germinal vesicle or polar body. This stereomicroscopic assessment was validated in an additional experiment. For this purpose, 222 cumulus-oocyte complexes were cultured for various periods. After denudation, the oocytes were divided into four groups, based on the observation of a germinal vesicle (GV), a polar body (MII) or neither of these (MI), or a degenerating appearance. Subsequently, the oocytes were mounted on slides and fixed in 75% ethanol/25% acetic acid for 24 h. The oocytes were stained with 1% aceto-orcein and the meiotic stage was determined by light microscopy (Table 1). Based on the results, rapid stereomicroscopic assessment sufficed to determine the nuclear status of oocytes in the present labelling experiments.

Table 1. Assessment of nuclear stage of cultured oocytes, before and after orcein staining

Nuclear status before staining	No. of oocytes	Nuclear stage after orcein staining			
		GV (%)	MI (%)*	MII (%)	Degenerate (%)
GV	51	48 (94)	3 (6)		
MI	96	2 (2)	94 (98)		
MII	71			70 (99)	1 (1)
Degenerate	4		1 (25)		3 (75)

*Including anaphase I and telophase I oocytes.

Electrophoretic analysis of oocyte proteins. One-dimensional electrophoresis was performed on 8–15% linear gradient SDS-polyacrylamide gels, according to Laemmli (1970). Fluorography was carried out by treating [³⁵S]methionine-containing gels with Amplify® (Amersham plc) for 30 min. All gels were dried under vacuum at 80°C and subsequently exposed to Kodak-XAR5 film. Mixtures of proteins, with known molecular weights, *M_r* 14 200–205 000 (Sigma Chemical Co., St Louis, MO, USA), were run simultaneously as standards, and visualized after electrophoresis by staining with Coomassie blue.

Results

Protein synthesis during maturation

To correlate the protein synthesis pattern with the nuclear stage, approximately 200 bovine cumulus-oocyte complexes were radiolabelled with [³⁵S]methionine at different times of culture.

The protein synthesis patterns were highly reproducible and representative patterns are shown in Fig. 1. Striking changes in the pattern occurred after 4 h of culture. Oocytes showing such a distinct protein synthesis pattern had undergone GVBD, as no intact germinal vesicle was ever observed in these oocytes. The protein synthesis patterns of 0 and 4 h exclusively represented oocytes possessing an intact germinal vesicle. Oocytes remaining in the germinal vesicle stage at 8 or even at 12 h of culture also showed such a pattern. Furthermore, changes were observed after 12 h of culture, during the transition from metaphase I to metaphase II stage. These changes did not coincide with polar body extrusion, since no polar body was detected in the oocyte cultured for 16 h, i.e. no changes were observed in the protein synthesis patterns after polar body extrusion had occurred (Fig. 1, 20 h).

Fig. 1. Protein synthesis patterns after 0, 4, 8, 12, 16 and 20 h of culture. Each lane is a representative pattern of a group of at least 20 individually labelled oocytes. A germinal vesicle was determined in the 0- and 4-h cultured oocytes and an extruded first polar body in the 20-h cultured oocyte. In the other oocytes, neither of these were observed. Striking changes in the protein synthesis between 4 and 8 h of culture, at M_r 175 000, 130 000, 110 000, 54 000, 42 000, 33 000, 30 000, 28 000, 15 000 and 14 000 (large arrows), and between 12 and 16 h of culture, at M_r 116 000, 95 000, 80 000, 26 000 and 22 000 (small arrows) are indicated.

Protein phosphorylation during maturation

Heavily phosphorylated proteins (M_r 19 000, 24 000 and two between 50 000 and 60 000) were mainly observed in the phosphorylation patterns before GVBD, when the same 4-h culture intervals were chosen as described above. A sudden increase in labelling was observed, especially after

4 h of culture. However, in these patterns, slight differences in the M_r of the two intensively phosphorylated proteins between 50 000 and 60 000 were detected. To examine these differences more precisely, about 300 cumulus-oocyte complexes were used to study the protein phosphorylation on a 1-h basis, from 0 up to 9 h of culture. The sequential phosphorylation patterns (Fig. 2) demonstrate that the phosphoprotein bands between 50 000 and 60 000 were dominantly displayed in patterns of oocytes cultured for 3 to 7 h and which were still in the germinal vesicle stage. Thereafter, these bands were less dominant. The phosphoprotein band at M_r 24 000 was also observed from 3 h of culture but this band stayed detectable in patterns of oocytes cultured for up to 16 h. The phosphoprotein with apparent M_r 19 000 was exclusively observed in the patterns of oocytes with an intact germinal vesicle, i.e. mainly from 0 to 7 h. Less obvious changes in the phosphorylation patterns preceding GVBD were found at M_r 29 000 and 130 000, whereas no changes were observed in the phosphorylation patterns after GVBD.

Fig. 2. Protein phosphorylation patterns of bovine oocytes cultured for successive 1-h periods, throughout 9 h of culture. Each lane is a representative pattern of a group of at least 20 individually labelled oocytes. After radiolabelling, oocytes were assessed for the presence (+GV) or absence (-GV) of a germinal vesicle. The dominant changes at M_r 19 000, 24 000 and between 50 000 and 60 000, and some minor changes at about 29 000 and 130 000, are marked by arrows.

Discussion

The present study demonstrates that during bovine oocyte maturation changes in protein synthesis exclusively take place after GVBD, whereas extensive phosphorylation of some proteins mainly occurs before GVBD. Despite the necessity of mRNA and protein synthesis to achieve GVBD (Hunter & Moor, 1987), the synthesis of specific proteins before GVBD, as reported for ovine oocytes (Moor & Crosby, 1986), was not detected. The most obvious changes are observed shortly

after GVBD, at the beginning of metaphase I, whereas the disappearance of several polypeptide bands occurs before polar body extrusion. These changes may occur at the end of metaphase I, during the transition to anaphase I/telophase I (Sirard *et al.*, 1989). Previously, extensive changes in protein synthesis after GVBD have also been demonstrated for oocytes of other mammalian species (mouse: Schultz & Wassarman, 1977; rabbit: Van Blerkom & McGaughey, 1978; sheep: Moor *et al.*, 1981). In addition, similar changes occur in enucleated oocytes, indicating that the 'reprogramming' of protein synthesis is not dependent on GVBD (Schultz *et al.*, 1978; Sun & Moor, 1988). However, in this study, oocytes remaining in the germinal vesicle stage exhibited a consistent protein synthesis pattern, even when oocytes were exceptionally retained in the GV stage after 8 or 12 h of culture. So far, aberrant protein synthesis patterns of GV-containing oocytes are only displayed by degenerate oocytes (Kastrop *et al.*, 1990).

The extensive phosphorylation of some proteins (M_r 24 000 and between 50 000 and 60 000) is observed from 3 h after the onset of culture. In addition, a phosphoprotein M_r 19 000 is exclusively present in patterns of oocytes remaining in the germinal vesicle stage. In spite of the slight molecular weight changes of the two phosphoproteins in the 50 000–60 000 region, the phosphorylation patterns after a specific culture period are very consistent. The slight differences could be caused by the phosphorylation of different proteins, or by different phosphorylation forms of the same protein(s) (Chen *et al.*, 1989). Our results are in favour of the latter possibility, as only slight differences in the molecular weights were detected and the changes occurred consistently during maturation. The heavily phosphorylated proteins may play a role in the activation of the maturation promoting factor (MPF) or as putative MPF substrates, since in *Xenopus* oocytes, a tremendous increase in phosphoproteins has been demonstrated to occur shortly before GVBD and in association with the activity of MPF (Maller & Smith, 1985). The exact function of these phosphoproteins during bovine oocyte maturation is the subject of further study.

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