Electron microscope observations on LH-induced oocyte maturation in Japanese quail (*Coturnix coturnix japonica*)

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The aim of this study was to describe the temporal sequence of ultrastructural changes in the boundary between the preovulatory oocyte and its surrounding follicular wall during maturation induced by injection of LH. Female Japanese quail were injected with ovine LH (20 µg per bird) 10–12 h before the expected time of ovulation. The largest and second largest follicles were excised before or 1, 2, 4 or 6 h after injection. The oocyte and the surrounding follicular wall were processed for observations using light and electron microscopy. Before injection of LH, cytoplasmic projections of granulosa cells interdigitated with microvilli on the surface of the oocyte and formed spot desmosomes and gap junctions with the oolemma. Two hours after injection of LH, the germinal vesicles in the largest but not in the second largest follicles began to break down and membrane-bound vesicles increased in number and size in the surrounding germinal disc. The junctions between the oocyte surface and the granulosa cell projections started to dissociate and a perivitelline space began to develop, possibly as the result of an accumulation of fluids transported from the capillary sinus in the theca interna. The first maturation spindle was formed 4 h after injection of LH, whereas the first polar body and the second maturation spindle were formed 6 h after LH stimulation. These observations suggest that the dissociation of connections between the oocyte and granulosa cells 2 h after exposure to increased concentration of LH is the first process of oocyte maturation. The associated increase in number and enlargement of membrane-bound vesicles in the germinal disc may be involved in the activation of factors involved in oocyte maturation.

Introduction

The germinal disc of the avian oocyte is a thickened mass of protoplasm in the centre of which lies the germinal vesicle adjacent to the oolemma (Olsen and Fraps, 1950). Oocyte maturation is induced by LH in birds (Olsen and Fraps, 1950) as in mammals and lower vertebrates (Lindner *et al.*, 1974; Tsafiri *et al.*, 1982; Patino and Thomas, 1990). Germinal vesicle breakdown occurs in the chicken approximately 6 h before the expected time of ovulation (Olsen and Fraps, 1950), the time when the LH concentration in the blood increases (Johnson and van Tienhoven, 1980). Condensation of chromatin, extrusion of the first polar body and formation of the second maturation spindle are completed 2.5 h before the expected time of ovulation (Olsen and Fraps, 1950). In quail the interdigitations between the surface membrane of the preovulatory oocyte and its surrounding granulosa cells disintegrate approximately 6 h before the expected time of ovulation, and the first polar body and second maturation spindle appear 1 h before the expected time of ovulation during spontaneous oocyte maturation (Yoshimura *et al.*, 1993). In mammals, the immature oocyte forms gap junctions with surrounding cumulus cells, which break down during oocyte maturation (Hyttel *et al.*, 1986;

Phillips and Dekel, 1991). There is no evidence for gap junctions between the oocyte and surrounding granulosa cells in birds, although open cytoplasmic communications have been described during the prelampbrush stage in quail follicles (Callebaut, 1991), as is the case in squamate reptiles (Andreuccetti *et al.*, 1978). In the quail, a few hours before ovulation, smooth membrane-bound vesicles are observed in the germinal disc in association with the formation of the second maturation spindle and the first polar body (Yoshimura *et al.*, 1993). The development of these membrane-bound vesicles may be related to the activation of the factors involved in oocyte maturation.

The precise temporal sequence of the ultrastructural changes at the interface between the preovulatory avian oocyte and the follicular wall after LH stimulation has not been described. In particular, details of germinal vesicle breakdown have not been observed because it is difficult to obtain the follicles at the appropriate stage of maturation during a naturally occurring preovulatory release of LH. In this study, the temporal sequence of the ultrastructural changes between the preovulatory oocyte and its surrounding follicular wall were observed before and after administration of LH. Particular note was taken of changes in the junctions between the oocyte and surrounding granulosa cells, the features of germinal vesicle breakdown and the development of membrane-bound vesicles in the germinal disc.

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Materials and Methods

Female Japanese quails laying five eggs or more in a sequence were maintained in individual cages at a lighting cycle of 14 h light (from 06:00 h to 20:00 h) and 10 h darkness, and provided with food and water *ad libitum*. Ovulation occurs 30–60 min after oviposition and approximately 6 h after the preovulatory release of LH (Doi *et al.*, 1980). Birds that had the second egg of a sequence in their shell glands were intravenously injected with ovine LH (20 µg per bird) or vehicle 10–12 h before the expected time of ovulation (06:00 h). LH (NIADDK-oLH-26) was dissolved in PBS (pH 7.4) at a concentration of 200 µg ml⁻¹. Birds were killed by decapitation before or 1, 2, 4 or 6 h after LH or vehicle injection. Each experimental group consisted of 4–7 birds. Immediately after death, the largest and second largest follicles were removed and processed for histological examination. Follicles were fixed in Karnovsky's fixative (4% paraformaldehyde and 5% glutaraldehyde in Millonig's buffer consisting of 0.12 mol NaH₂PO₄ l⁻¹ and 0.12 mol NaOH l⁻¹, pH 7.2) at 4°C for 1 h. The germinal disc region of the oocyte and associated follicular wall were then cut and refixed overnight in the same fixative at 4°C. These tissues were then washed with Millonig's buffer for 2 h, followed by post-fixation in 1.3% osmium tetroxide in Millonig's buffer at 4°C for 2 h. Tissues were then dehydrated with acetone and embedded in Epon 812. For light microscope observations thick sections were stained with toluidine blue. Thin sections were stained with uranyl acetate and lead acetate, and examined under JEM 1200EX electron microscope (JEOL, Japan) at an accelerating voltage of 60 kV.

Results

Before injection of LH the germinal vesicle was located in the centre of the germinal disc adjacent to the oolemma and surrounded with dense protoplasm (Fig. 1a). The germinal vesicle was bounded by a smooth membrane and contained an amorphous matrix with spherical postlampbrush chromosomes (Callebaut, 1973) (Fig. 1b). The oocyte was surrounded by a perivitelline layer and follicular wall consisting of a granulosa layer, theca interna and theca externa (Fig. 1b). No significant structural changes were observed in the germinal vesicle 1 h after injection of LH. Two hours after injection of LH, the germinal vesicle had shrunk and its membrane had become folded. The cytoplasm surrounding the residue of the germinal vesicle contained no yolk granules but was filled with a lightly stained matrix which may have leaked out of the vesicle (Fig. 2a). Spherical postlampbrush chromosomes were localized in the centre of the degenerating germinal vesicle (Fig. 2b). The first maturation spindle was located perpendicular to the surface of the germinal disc 4 h after LH injection (Fig. 3a). Six hours after LH injection, the first polar body was located in the perivitelline space and the second maturation spindle was observed near the surface of the oolemma (Fig. 3b).

Observations using the electron microscope showed that before injection of LH the germinal vesicle contained a lightly stained amorphous matrix surrounded by distinct inner and outer nuclear membranes (Fig. 4). Numerous ooplasmic elements

including membrane-bound vesicles, oval or elongated mitochondria, Golgi apparatus, glycogen granules and yolk spheres were observed in the protoplasm adjacent to the germinal vesicle (Fig. 4). In the germinal disc distant from the germinal vesicle, numerous membrane-bound vesicles were located near the oolemma with yolk spheres accumulated under them. Cytoplasmic projections of the granulosa cells interdigitated with microvilli on the surface of the oocyte and formed spot desmosomes and gap junctions with the oolemma. The spot desmosomes were characterized by dense plaques just beneath the granulosa cell plasma membrane and oolemma (Fig. 5a), whereas gap junctions were formed by the close apposition of the granulosa cell plasma membrane and oolemma separated by a narrow gap (Fig. 5b).

Two hours after injection of LH, ultrastructural changes were observed in the oocyte and follicular wall (Fig. 6). Vacuoles were associated with the germinal vesicle membrane and some parts of it were disintegrating. A small number of dense bodies, membrane-bound vesicles and glycogen granules were scattered around the germinal vesicle (Fig. 6). Membrane-bound vesicles were observed in the germinal disc distant from the germinal vesicle that were not observed here before injection of LH (Fig. 7). In four of seven preovulatory follicles, the granulosa cells and the oocyte surface became less tightly associated as shown by development of a wide perivitelline space between the perivitelline membrane and oolemma (Fig. 7). Many oocyte microvilli were erect and not associated with granulosa cell projections. In some cases, gap junctions were still present between the large cytoplasmic processes of granulosa cells and the oolemma (Fig. 7).

Four hours after LH injection, the membrane-bound vesicles located beneath the oolemma in the germinal disc were greatly distended (Fig. 8). Spot desmosomes and gap junctions between oocyte and granulosa cell membranes were not observed in any of the follicles and were assumed to have disintegrated.

No significant structural changes were observed in oocytes in the second largest follicle of birds injected with LH or in the largest and second largest follicles from birds injected with vehicle.

Discussion

The present results demonstrate that injection of quail with LH 10–12 h before ovulation induces oocyte maturation in the largest follicle but not in smaller follicles. This result supports the report that LH is the primary factor involved in the induction of oocyte maturation in birds (Olsen and Fraps, 1950). However, it remains unclear why only the oocyte in the largest follicle undergoes maturation. In lower vertebrates, progesterone or other steroid hormones produced in the granulosa cells in response to LH may induce oocyte maturation through interaction with specific steroid receptors in the oolemma (Haider, 1990). In chickens, granulosa cells in the mature preovulatory follicle, in contrast to those in smaller follicles, produce large amounts of progesterone in response to LH (Nakamura *et al.*, 1991). The granulosa cells in the largest quail follicle contain prominent paranuclear lipid droplets (Callebaut, 1988; Callebaut *et al.*, 1991), suggesting that they have a steroidogenic function (Van Nassauw and Callebaut, 1991). If progesterone is one of

Fig. 1. (a) Light micrograph of a cross-section of the germinal disc of a preovulatory follicle from the Japanese quail 10 h before the expected time of ovulation and before LH injection. The germinal vesicle (GV) is located in the centre of the germinal disc (GD). FW: follicular wall. Scale bar = 50 µm. (b) Magnified view of the section shown in (a). The follicular wall consists of the granulosa layer (G), theca interna (I) and theca externa (E). Scale bar = 25 µm. Inset shows the postlampbrush chromosomes in the centre of the germinal vesicle. Scale bar = 10 µm.

Fig. 2. (a) Light micrograph of a cross-section of the germinal disc 2 h after LH injection. The germinal vesicle (GV) is breaking down. Note the folds of the germinal vesicle membrane. Scale bar = 25 µm. (b) Light micrograph of a cross-section of the germinal vesicle 2 h after LH injection. The postlampbrush chromosomes (arrow) are still observed in the centre of the germinal vesicle. Scale bar = 10 µm.

Fig. 3. (a) Light micrograph of a cross-section of the germinal disc 4 h after LH injection. Note that the first maturation spindle which contains chromosomes is formed (arrow). Scale bar = 10 µm. (b) Light micrograph of a cross-section of the germinal disc 6 h after LH injection. Note the second maturation spindle (arrow) and the first polar body (arrowhead). Scale bar = 10 µm.

Fig. 4. Electron micrograph of the germinal disc and germinal vesicle 10 h before the expected time of ovulation and before LH injection. Note that the membrane-bound vesicles (arrow) and numerous cytoplasmic organelles are distributed near the germinal vesicle (GV). Y: yolk sphere. Scale bar = 1 μ m.

Fig. 5. (a) Electron micrograph of a junction between the granulosa cell projection and the oocyte. Note the close contact of the large cytoplasmic projection of the granulosa cell (P) with the oocyte (arrow). O: oocyte; PV: perivitelline layer. Scale bar = 200 nm. (b) Magnified view of the section shown in (a). Arrow indicates that the close contact of the granulosa cell projection (P) and oolemma is the gap junction. O: oocyte. Scale bar = 50 nm.

Fig. 6. Electron micrograph of the germinal disc 2 h after LH injection. Note the germinal vesicle (GV) which is breaking down. The germinal vesicle membrane (long arrow) shows an irregular appearance, and the vacuoles formed are associated with the germinal vesicle membrane, suggesting that the membrane is digested. Short arrows indicate membrane-bound vesicles that are highly developed compared with those observed before LH injection (Fig. 4). GD: germinal disc. Scale bar = 1 μ m.

Fig. 7. Electron micrograph of the germinal disc distant from the germinal vesicle 2 h after LH injection. Note that a wide perivitelline space (S) is formed and the junctions of the granulosa cell projections with the oocyte are dissociated except for a few gap junctions which remain attached (long arrows). Short arrows indicate the developing membrane-bound vesicles. Scale bar = 1 μ m.

Fig. 8. Electron micrograph of the germinal disc 4 h after LH injection. Note the markedly developed membrane-bound vesicles (arrows) in the germinal disc. Scale bar = 1 μ m.

the hormones that controls oocyte maturation in hens, the ability of the largest follicle to secrete copious amounts of progesterone may be one of the reasons why LH induces maturation of the oocyte only in the largest follicle.

It was observed that projections of the granulosa cells form interdigitations, spot desmosomes and gap junctions with the oocyte surface before LH injection. The interdigitations and spot desmosomes between them have been described by Perry *et al.* (1978a, b) and Yoshimura *et al.* (1993), but this is the first report on a bird that demonstrates the presence of gap junctions. The gap junctions between the oocyte and granulosa cells suggest metabolic coupling between them. Two hours after LH stimulation, these junctions had dissociated with only a few gap junctions remaining in four of the seven follicles examined. This observation confirms our earlier finding that interdigitations between the granulosa cell projections and the preovulatory oocyte break down 6 h before the expected time of ovulation (Yoshimura *et al.*, 1993). In mammals also, gap junctions between the oolemma and granulosa cells in immature follicles break down during oocyte maturation (Gilula *et al.*, 1978; Hyttel *et al.*, 1986). In mice, channels in gap junctions between the cumulus cell projections and the oocyte may be blocked even though the junctions are not dissociated morphologically during oocyte maturation (Phillips and Dekel, 1991). In the present study, three of the seven follicles did not show significant morphological changes in the junction between the granulosa cells and oocyte 2 h after LH injection. Observations by Phillips and Dekel (1991) suggest that the functional coupling of the granulosa cells with the oocyte may have been disrupted in the absence of any visible morphological change in these three follicles.

Studies in mammals suggest that cumulus and granulosa cells produce factors that prevent oocyte maturation after transfer to

the oocyte via gap junctions with these cell types (Sato and Koide, 1984; Anderson *et al.*, 1985; Sirard and Bilodeau, 1990). The metabolic coupling between the cumulus cells and oocyte via gap junctions is terminated at the time of oocyte maturation resulting in the resumption of meiosis (De Loos *et al.*, 1991). It is therefore possible that, in quail, metabolic coupling between granulosa cells and the oocyte may prevent oocyte meiosis in immature follicles. The interruption of this coupling between granulosa cells and the preovulatory oocyte may be the first step in oocyte maturation that occurs during the 2 h after LH stimulation.

The formation of a wide perivitelline space between the maturing oocyte and surrounding granulosa cells after LH stimulation may be induced by an influx of blood serum components from the theca interna (Yoshimura *et al.*, 1993). In mammals, the preovulatory LH surge is thought to induce hyperaemia and increase permeability of the thecal capillary sinus (Espey, 1980). It is possible that LH has a similar action in quail.

Maturation-promoting factor activity is believed to play a key role in the progression of the cells from interphase to metaphase in both meiosis and mitosis (Masui and Markert, 1971; Nurse, 1990) including the process of oocyte maturation (Motlik and Kubelka, 1990). Inactive maturation-promoting factor contained in the immature ooplasm is activated when the oocyte resumes meiosis (Motlik and Kubelka, 1990). In an immunoblotting experiment for maturation-promoting factor in the ovary of quail, Mori *et al.* (1991) found two bands representing 32 and 34 kDa proteins in immature and mature oocytes. A third band, a protein of approximate molecular mass of 32 kDa, which may be an active form of maturation-promoting factor, was found exclusively in the oocyte after germinal vesicle

breakdown. The present study showed that membrane-bound vesicles were developing in the germinal disc as the germinal vesicle was breaking down 2 h after LH injection and that these vesicles were markedly developed after germinal vesicle breakdown 4 h after LH injection. Development of the membrane-bound vesicles in the germinal disc also occurs during spontaneous oocyte maturation (Yoshimura *et al.*, 1993). The temporal correlation between the changes of maturation-promoting factor activity and the development of the membrane-bound vesicles suggests that the development of these vesicles may be related to the activation of maturation-promoting factor.

The present results indicate that germinal vesicle breakdown occurs approximately 2 h after LH stimulation. The appearance of vacuoles associated with germinal vesicle membrane breakdown suggests that the membrane may be digested. However, factors responsible for the digestion of germinal vesicle membrane remain unknown. Although it is suggested that maturation-promoting factor or some phosphoproteins are responsible for induction of germinal vesicle breakdown (Thibault *et al.*, 1987; Rime and Ozon, 1990; Haider, 1990; Kang *et al.*, 1991), no evidence has been obtained that these proteins digest the germinal vesicle membrane. It is reported that proteinase activity participates in the process of spindle formation (Hashimoto *et al.*, 1990), but there is no evidence indicating that proteinase activities are involved in the breakdown of the germinal vesicle membrane.

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