Time-Related Morphological Changes of Porcine Ovarian Granulosa Cells Incubated with Urea-EDTA Solution

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ABSTRACT. Morphological profiles of porcine granulosa cells incubated with 10 mM Tris-HCl containing 1 M urea and 5 mM EDTA (urea-EDTA solution) were investigated. The percentages of granulosa cells incorporating the dye, trypan blue on incubation with urea-EDTA solution did not change during the initial 30 min. Thereafter, granulosa cells gradually took up the dye throughout the incubation. The amount of protein released from granulosa cells increased dramatically during initial 15 min of incubation, but decreased during the following 15–30 min of incubation. Thereafter, the amount of proteins released from granulosa cells increased gradually again. The releasing profile of ^{51}Cr -bounded substances in granulosa cells increased markedly during the initial 15 min of incubation, and decreased during the next 15 min of incubation. Subsequently the amount of ^{51}Cr released was enhanced. The plasma membranes of granulosa cells remained intact at 30 min of incubation, although chromatin clusters of granulosa cells disappeared. Thereafter, a number of cells showed signs of degeneration, including broken plasma membrane and cytolysis. The present study revealed that urea-EDTA solution is useful in extracting materials from porcine granulosa cells. The majority of the materials extracted from granulosa cells during the initial 30 min of incubation with urea-EDTA solution is considered to be from the cell surfaces and/or intercellular matrix.—**KEY WORDS:** granulosa cell, porcine ovary, urea-EDTA.

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The cell surface is of special interest in studies of cell differentiation because it forms the boundary at which interactions between the cell and its environment occurs. In particular, it is now well established that cellular recognition is mediated in whole or in part by the cell membrane and that cell surface mediated events play an important role in various developmental systems [9]. Much of the environmental sensing performed by cells is mediated through proteins or glycoprotein receptors intercalated into the cell membrane [5]. Then identification of cell surface materials is important in the biochemical analysis of cell function.

A cell aggregation factor was extracted by dissociating sea urchin embryo with 10 mM Tris-HCl containing 1 M urea and 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.3) (urea-EDTA solution) [14]. The substances of cell surfaces of cultured fibroblasts were extracted by the same solution, and the investigation proved that urea-EDTA solution is also useful for the extraction of cell surface or intercellular materials of higher animals with minimum destruction of cells, because urea-EDTA solution is nearly isotonic with the cells [13].

In ovarian follicles, the major cellular elements are granulosa cells [11, 17]. Granulosa cells secrete many biologically active substances [1, 6–8, 19]. Some of the substances secreted by granulosa cells

are supposed to localize on the cell surface or in the intercellular spaces [8, 19]. In the present study, morphological alterations of granulosa cells induced by urea-EDTA treatment were determined to establish that majority of the materials extracted with urea-EDTA solution were from the cell surface or the extracellular matrix.

MATERIALS AND METHODS

Animals and cell preparations: Porcine ovaries were obtained from non-cycling, prepubertal gilts of approximately 100 kg body weight at a local slaughterhouse. The ovaries were excised within 30 min after sacrifice and placed in 0.85% physiological saline solution containing penicillin (100 IU/ml) and streptomycin (50 $\mu\text{g}/\text{ml}$) at 35°C. The granulosa cells (or granulosa cell layer) were obtained by needle aspiration from medium-sized (2–5 mm in diameter) follicles.

Treatment of granulosa cells: Granulosa cells obtained as mentioned above were separated from follicular fluid by centrifugation and washed with 0.85% physiological saline solution five times, and then 1 ml suspension of granulosa cells (5×10^8 cells) was incubated with 10 ml of 10 mM Tris-HCl buffer containing 1 M urea and 5 mM EDTA (pH 8.3) at 37°C for varying duration. After settling, the

cells were removed by centrifugation at 10,000 g, at 4°C for 30 min. Spun-downed cell pellets were sonicated and DNA content determined by method of Burton [4]. The supernatant (10 μ l) was collected and protein concentration measured. Protein was determined by the method of Lowry *et al.* [15] using crystalline bovine serum albumin as standard. The results were expressed as released protein per 100 μ g DNA of incubated granulosa cells.

Dye exclusion test for viability: One volume of 1% trypan blue solution in physiological saline solution and one vol. of granulosa cell suspension were mixed and incubated for 2 min at room temperature. The number of stained granulosa cells was counted under a light microscope.

^{51}Cr -labelled cells: 5×10^7 granulosa cells in 1 ml physiological saline solution were incubated for 30 min at 37°C with 0.2 ml sodium chromate (^{51}Cr , specific activity 400–1,200 Ci/g chromium, Du Pont, France) as described by Brunner *et al.* [3]. After incubation the cells were washed three times with physiological saline solution and diluted to the required concentration.

Cytolytic assay: 1.5×10^6 ^{51}Cr -labelled granulosa cells were incubated in 2 ml of urea-EDTA solution. Incubation was carried out for varying periods of time at 37°C. Incubation was followed by centrifugation at 2,000 g for 5 min, after which an aliquot (10 μ l) of cell-free supernatant was removed and radioactivity determined in an Auto-Gamma Spectrometer (Packard). The rate of specific release of ^{51}Cr from the cells was calculated by subtracting the spontaneous release from the total release. The spontaneous release increased from 6% at the beginning to 8% at the end of the 2 hr incubation period. The results were expressed as released ^{51}Cr (cpm) per 10 μ g DNA of incubated granulosa cells.

Electron microscopy: A total of 5×10^7 granulosa cells freshly dissociated from ovarian follicles, was incubated in 1 ml of urea-EDTA solution for 30, 60 and 90 min. After rinsing in physiological saline solution, the cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hr at 4°C, and were then washed with 0.1 M phosphate buffer (pH 7.4). The cells were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 2 hr at 4°C, and subsequently rinsed in the same buffer. They were dehydrated in a graded series of ethanol, and embedded in Luveak 812 (Nacalai tesque Co., Japan). Ultrathin sections were cut with a Sorvall Porter-Blum MT-1 ultramicrotome, mounted on

copper grids, stained with uranyl acetate and lead citrate and examined with Hitachi H-300 transmission electron microscope at 75 kV.

RESULTS

Dye exclusion of granulosa cells incubated with urea-EDTA solution: Figure 1 shows the percentages of granulosa cells incorporating the stain, trypan blue, after incubation in urea-EDTA solution for varying periods of time. More than 99% of granulosa cells after washing with 0.85% physiological saline solution were unstained and the percentages of stained cells did not change during the initial 30 min. Thereafter an increasing number of the granulosa cells suspended in urea-EDTA solution took up the stain. At 45 min, about 12% incorporated the stain and at 60 min, practically 78% had done so, an indication that after 30 min the treatment was detrimental to the granulosa cells. In contrast granulosa cells incubated for 120 min in 0.85% physiological saline solution excluded the dye.

Protein separated from granulosa cells: Figure 2 shows the amount of proteins extracted from granulosa cells at varying duration of incubation with urea-EDTA solution. Substantial amounts of pro-

Fig. 1. Dye exclusion of porcine granulosa cells incubated in saline (○) and in 10 mM Tris-HCl containing 1 M urea and 5 mM EDTA (●) for varying periods of time. After the granulosa cells were suspended in the media, they were incubated in 1% trypan blue for 2 min and examined for staining. Values are mean $\pm$ SD of five independent experiments.

Fig. 2. The amount of protein extracted from granulosa cells incubated for varying periods of time at 37°C in 10 mM Tris-HCl buffer containing 1 M urea and 5 mM EDTA. An aliquot of cell-free supernatant was removed and its protein concentration was determined. Values are mean $\pm$ SD of five independent experiments.

Fig. 3. Release of ⁵¹Cr from the labelled granulosa cells incubated for varying periods of time at 37°C in 10 mM Tris-HCl solution containing 1 M urea and 5 mM EDTA. An aliquot of cell-free supernatant was removed and its radioactivity was determined. Values are means $\pm$ SD five independent experiments.

teins was extracted from granulosa cells until 15 min of incubation. Only a small amount was extracted during the initial 15 min to 30 min of incubation. At 30 min of incubation, about 0.32 mg of protein was extracted from a standard volume of granulosa cells (100 µg DNA). Thereafter, the amount of extracted proteins increased gradually.

Specific release of ⁵¹Cr from the labelled granulosa cells: The specific release of ⁵¹Cr from the labelled granulosa cells was measured at every 30 min for 3 hrs. Figure 3 indicates cpm derived from 10 µg DNA of granulosa cells. At the initial 15 min about 26% of ⁵¹Cr-bounded to granulosa cells was released in urea-EDTA solution, and thereafter the percentage of released ⁵¹Cr did not change until 30 min of incubation. Between 60–120 min of incubation, about 50% of labelled ⁵¹Cr was extracted from the granulosa cells. At 180 min of incubation, about 60% of ⁵¹Cr was removed.

Ultrastructure of granulosa cells incubated with urea-EDTA solution: Freshly dissociated cells were oval in shape. Occasional microvilli and a few blebs were visible on the cell surfaces (Fig. 4A). Clusters of chromatin were seen in the nucleus. Cells maintained in urea-EDTA solution for 30 min increased in size compared with that of untreated cells, and exhibited smooth surfaces that were almost devoid of microvilli (Fig. 4B). Chromatin clusters disappeared and nucleus became round. However, plasma membranes remained intact. At 60 min of incubation, the cells showed signs of degeneration (Fig. 4C). Broken plasma membranes, indicative of cytolysis, were found. Cytolysis of large numbers of granulosa cells occurred from 60 to 90 min of incubation (Fig. 4C, D). Cytoplasmic organelles escaped from the cells, and filled the intercellular space of the granulosa cells (Fig. 4D).

DISCUSSION

The majority of follicles in the ovary will undergo atresia [21]. It is estimated that about 50–75% of antral follicles are atretic [2, 16, 18]. Atresia eventually affects all components of the antral follicles: oocyte, granulosa cells and theca layer [21]. In the histological section, dead granulosa cells having pycnotic nuclei are observed in follicles progressing to atresia [10, 12, 20]. It is presumed that there was a significant number of dead cells among the granulosa cells separated from porcine antral follicles in this series of experiments. However, more than 99% of granulosa cells did not stain with the dye, trypan blue. These results suggest that dead granulosa cells were eliminated during the washing 5 times with physiological saline solution.

The results of the dye exclusion test (Fig. 1) suggest that the cell membrane remains intact when the granulosa cells were incubated in urea-EDTA

Fig. 4. Morphological changes of porcine granulosa cells incubated for 0 (A, control), 30(B), 60(C) and 90(D) min in 10 mM Tris-HCl solution containing 1 M urea and 5 mM EDTA. $\times 5,000$. Note intact membranes of cells maintained in urea-EDTA solution for 30 min (B), and cells of 60, 90 min of incubation showing signs of degeneration (C, D).

solution providing treatment time was shorter than 30 min. Moreover, the dye exclusion of the granulosa cells during this brief period of treatment was verified by morphological observations.

Releasing profile of ^{51}Cr bound to granulosa cells is considered to reflect the extracting profile of the materials from cells [3]. At the initial 15 min, about 26% of ^{51}Cr bound to granulosa cells was released during incubation in urea-EDTA solution, and up to the initial 30 min of incubation, the released amount had not changed. After that the amount released was markedly enhanced. Releasing profile of protein from granulosa cells in urea-EDTA solution confirmed the results using ^{51}Cr -labelled granulosa cells. These results suggest that the material located on the cell surfaces and intercellular spaces were

extractable up to 30 min of incubation in urea-EDTA solution. Subsequently, the cell became fragile that intracellular materials were extracted. Taken together, it is suggested that the majority of the material extracted from granulosa cells during the initial 30 min of incubation with urea-EDTA solution is from the cell surfaces and/or intercellular matrix.

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