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Differential effects of mitochondrial inhibitors on porcine granulosa cells and oocytes

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ABSTRACT

Oocytes and granulosa cells rely primarily on mitochondrial respiration and glycolysis for energy production, respectively. The present study examined the effect of mitochondrial inhibitors on the ATP contents of oocytes and granulosa cells. Cumulus cell-oocyte complexes (COCs) and granulosa cells (GCs) were collected from the antral follicles of porcine ovaries. Treatment of denuded oocytes with either carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), antimycin, or oligomycin significantly reduced ATP content to very low levels (CCCP, 0.12 pM; antimycin, 0.07 pM; and oligomycin, 0.25 pM; $P < 0.05$), whereas treatment with a glycolysis inhibitor (bromopyruvic acid, BA) had no effect. Conversely, the ATP content of granulosa cells was significantly reduced by treatment with the glycolysis inhibitor but was not affected by the mitochondrial inhibitors (ATP/10,000 cells; control, 1.78 pM and BA, 0.32 pM; $P < 0.05$). Reactive oxygen species (ROS) generation after CCCP treatment was greater in oocytes (1.6-fold) than that seen in granulosa cells (1.08-fold). Oocytes surrounded by granulosa cells had higher ATP levels than denuded oocytes. Treatment of COCs with CCCP reduced, but did not completely abolish, ATP content in oocytes (control, 3.15 pM and CCCP, 0.52 pM; $P < 0.05$), whereas treatment with CCCP plus a gap junction inhibitor, 18α-glycyrrhetinic acid, and CCCP decreased the ATP content to even lower levels (0.29 pM; $P < 0.05$). These results suggest that granulosa cells are dependent on glycolysis and provide energy to oocytes through gap junctions, even after treatment with CCCP.

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1. Introduction

Oocyte growth is dependent on communication with granulosa cells (GCs), with the latter providing energy to the former [1]. In this context, it has been shown in pigs and cows that the number GCs and cumulus cells that surround an oocyte is closely related to the energy status of the enclosed oocytes, as determined by ATP levels, lipid content, and acetylation levels [2,3]. Furthermore, in mice, denuded oocytes were found to have a lower ATP content than those enclosed by cumulus cells [4]. Oocytes depends on oxidative phosphorylation, which is supported by the observation that oocytes preferentially utilize pyruvate and have low phosphofructokinase activity [5,6]. In contrast to oocytes, GCs are thought to rely mainly on glycolysis for energy, and deliver the metabolites derived from glucose to oocytes via gap junctional communication [7,8]. Furthermore, high granulosa cellular glycolytic activity is important for follicle development [9]. Mitochondria are important organelles for energy productions. Low mitochondrial counts have been found to have a pronounced effect on oocyte viability, and low oocyte ATP levels are associated with low developmental competency [10–12]. Furthermore, photosensitization-induced mitochondrial dysfunction in oocytes affected their maturation and development [13,14]. Maternal physiological conditions are known to affect mitochondrial quality. For example, aging affects the quality and quantity of mitochondria in oocytes as well as in somatic cells [15–18]. However, the precise mechanism by which mitochondrial dysfunction affects the energy levels of GCs, denuded oocytes, and oocytes surrounded by cumulus cells remains unclear. Mitochondrial activity can be blocked by several different mechanisms, including mitochondrial membrane uncoupling (e.g., carbonyl cyanide *m*-chlorophenyl hydrazine, or CCCP), inhibition of mitochondrial electron transport complex III (e.g., antimycin), and inhibition of mitochondrial ATP synthase (e.g., oligomycin). Interestingly, it has

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been reported that the poor response of GCs to mitochondrial uncoupling is related to the developmental competency of oocytes [19]. This put forth the hypothesis that GCs depend mainly on glycolysis and can therefore provide oocytes with energy even in the presence of mitochondrial inhibitors. Here, we examined the mechanism by which mitochondrial inhibitors affect the energy status of GCs and oocytes, and investigated the contribution of GCs to the energy status of oocytes cultured in the presence of mitochondrial inhibitors.

2. Materials and methods

2.1. Chemicals and reagents

CCCP (10 μM), antimycin (5 μM), oligomycin (2.5 μM), the gap junctional inhibitor 18a-glycyrrhetinic acid (18 GA, 50 μM), and the glycolysis inhibitor, bromopyruvic acid (BA, 0.5 or 5 mM), were diluted in DMSO (× 2000). Control medium was added at the same concentration as that of DMSO.

2.2. Oocyte and granulosa cell collection and culture

Ovaries were collected from gilts obtained from a local slaughterhouse and transported to the laboratory within 0.5 h. GCs were aspirated from 3 mm antrum follicles using a syringe connected to an 18-gauge needle. Cumulus cell-oocyte complexes (COCs) were separated from the follicular contents under a stereomicroscope, using a Pasteur pipette. The cellular suspension was strained through a 40-μm nylon mesh (BD Falcon, Bedford, MA, USA) to remove cellular debris, and centrifuged at 200 × *g*, for 1 min at 25 °C to obtain a pellet. GCs were dispersed using an enzyme cocktail (Accumax, Innovative Cell Technologies, San Diego, CA, USA) according to the manufacturer's instructions. Cells were resuspended in culture medium (TCM-199, 1 mM taurine, 100 IU/mL penicillin, 0.1 μg/mL streptomycin, 50 μg/mL gentamicin, and 5% fetal calf serum) and cultured in a 4-well dish (Nunc, Thermo Scientific, Waltham, MA, USA) at 2.5×10^5 cells/well for 24 h at 38.5 °C, with maximum humidity, under atmospheric conditions of 5% CO_2 and 95% air. After incubation, un attached GCs were removed by gentle pipetting and used for experimental analysis. Denuded oocytes were obtained by treating COCs with 0.1% hyaluronidase for 5 min followed by vortexing for 5 min.

2.3. ATP content

ATP content was measured using an ATP-dependent luciferin-luciferase reaction with an ATP assay kit (TOYO B-Net, Tokyo, Japan) according to the manufacturer's instructions, with minor modifications. To determine GC ATP content, culture medium was aspirated, GCs were washed twice with PBS and frozen in 100 μL of water on an aluminum block immersed in liquid nitrogen. Upon warming, cellular suspensions were collected from each well and divided into two equal-volume aliquots; one used for the ATP assay and the other for DNA extraction to determine cell number using real time PCR (see below). To determine oocyte ATP content, denuded oocytes were transferred individually into 50 μL of water and ATP content was measured.

2.4. Cell number determination

An equal volume of (2 ×) DNA extraction buffer (Tris-HCl, 40 mM; Nonidet-40 and Tween 20, 1.8%; and proteinase K, 0.8 mg/mL) was added to the cell suspension and heated to 55 °C for 30 min followed by 98 °C for 5 min. Copy number of GCG glucagon (a single-copy gene) was determined using real-time PCR with a Corbett Rotor Gene 6000 Real-Time Rotary Analyzer (Corbett Research, Sydney, Australia). Primers (5′- agcagaatcaacaccatcggt -3′ and 5′- tggctccacccatagaatgc -3′, 154 bp) were designed using the NCBI database (Refseq NC_010457), and Primer-BLAST. PCR was performed with an initial denaturation at 95 °C for 3 min followed by 40 cycles of 98 °C for 5 s and 59 °C for 11 s. SYBR green fluorescence was measured at the end of each extension step. A standard curve was generated for each run using 10-fold serial dilutions representing copies of the external standard. The external standard was the PCR product of the corresponding gene cloned into a vector using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Products were confirmed by sequencing prior to use. Duplicate measurements were conducted for each sample, and all amplification efficiencies were greater than 1.95.

2.5. Reactive oxygen species measurement

COCs or GCs were incubated in a medium containing CCCP or DMSO for 30 min. Cells were then incubated in TCM-199 medium containing reactive oxygen species (ROS) detection reagents (Invitrogen, OR, USA) for 30 min followed by a 30-min incubation in TCM-199 medium without reagents. Oocytes were examined under a fluorescent microscope (Keyence, Tokyo, Japan), and fluorescent intensities of CCCP groups and vehicle groups were compared. Approximately 20 oocytes were used for this comparison. GCs were counterstained with Hoechst 33342 before observation, and fluorescence intensity of the ROS reagent was normalized to the intensity of Hoechst. Comparisons were performed eight times using different cell batches.

2.6. Assay to determine the effect of mitochondrial and glycolytic inhibitors on denuded oocytes and GCs

Denuded oocytes or GCs were incubated in a medium (TCM-199) containing antimycin, oligomycin, CCCP, BP or vehicle (DMSO) for 30 min followed by determination of ATP content. Approximately 20 oocytes and GCs from four different batches were used in each treatment group.

2.7. Assay to determine the effect of CCCP treatment of COCs on oocyte ATP content

COCs were treated with CCCP, a gap junctional inhibitor (18 GA), or a combination of both for 30 min, and oocyte ATP content was then measured. To address whether the effectiveness of CCCP depends on the number of GCs, the experiment was repeated by using oocytes surrounded by thick cell layers (rich COCs) and oocytes surrounded by a thin layer (poor COCs), which were visually selected from pooled COCs (Fig. 1).

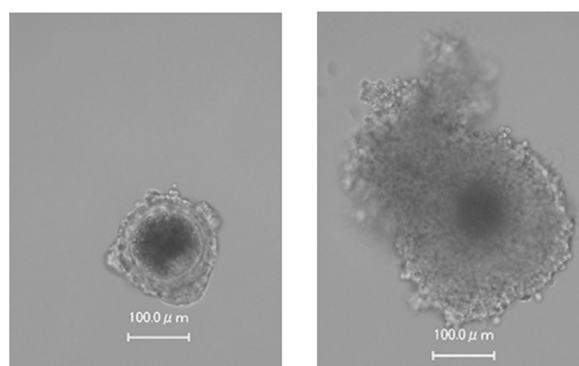

Fig. 1. Oocytes surrounded by a thick layer (A) and a thin layer (B) of granulosa cells.

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