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An intercellular pathway for glucose transport into mouse oocytes

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GLUCOSE IS AN ESSENTIAL NUTRIENT for mammalian cells. It is employed as an energy source via glycolysis and the tricarboxylic acid (TCA) cycle. Glucose can also be metabolized via the pentose phosphate pathway (PPP) to produce NADPH and ribose 5-phosphate for nucleotide biosynthesis. In addition, glucose can be used for the synthesis of amino acids, O-linked glycosylation, and production of substrates for extracellular matrixes (45). Glucose enters cells either by an active process via sodium-coupled glucose transporters (SGLTs) or through facilitative glucose transporters (GLUTs). Fourteen paralogs of the GLUT gene family have been identified, including GLUT1–12, the H+–coupled myoinositol transporter, and GLUT14 (24).

In mammalian antral follicles of the ovary, the oocyte is surrounded by numerous layers of granulosa cells (also termed cumulus cells) forming the cumulus-oocyte complex (COC). Oocytes are coupled to these companion somatic cells through extracellular matrix—specific gap junctions (1). Gap junctions consist of an array of intercellular channels that allow direct sharing of small molecules between the interconnected cells. Each intercellular channel consists of two hemichannels (connexons) that are docked end to end, with each cell contributing one connexon (27). Passage of ions, metabolites, amino acid, and signaling molecules etc., from granulosa cells to oocytes via gap junctions provides a physical basis for their metabolic cooperation (43).

Despite the suggested importance of glucose, there is so far no direct experimental evidence delineating how glucose is transported into the COC. In the present study, by using 6-NBDG ([6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-6-deoxyglucose]), a nonmetabolizable fluorescent glucose derivative, to trace glucose transport within cumulus-oocyte complexes (COCs), we employed high-magnification confocal microscopy to examine the distinct roles in glucose uptake by cumulus cells and the oocyte. We showed that fluorescent glucose enters both cumulus-enclosed and denuded oocytes. Pharmacological blockade of gap junctions between the oocyte and cumulus cells significantly inhibited fluorescent glucose transport to oocytes. Moreover, we found that both in vivo hyperglycemic environment and in vitro high-glucose culture increased free glucose levels in oocytes via gap junctional channels. These findings reveal an intercellular pathway for glucose transport into oocytes whereby glucose is taken up in cumulus cells via GLUTs and then transferred into the oocyte through gap junctions. This intercellular pathway may partly mediate the effects of high-glucose condition on oocyte quality.

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To collect ovulated metaphase II (MII) oocytes, mice received an injection of 10 IU human chorionic gonadotropin (hCG) 2 days after PMSG priming. Oocytes were recovered from oviduct ampullae 13.5 h after hCG, and cumulus cells were removed by incubating briefly in 1 mg/ml hyaluronidase.

Evaluation of glucose transport in COCs. 6-NBDG (Molecular Probes, Eugene, OR), a fluorescent glucose analog, was used to report glucose transport. In brief, COCs or denuded oocytes (DO) were incubated in M2 medium containing 200 μM NBDG, with the concentration chosen as the capable of giving an adequate signal-to-noise ratio. Following three rapid washes, live cells were immediately imaged at 488 nm by fluorescence microscope (Zeiss Axioskop, Göttingen, Germany). Fluorescence signal was quantified using NIH Image J software and then was calculated as the average intensity after background subtraction.

Glucose competition assay. To test the competitive cellular uptake of 6-NBDG, COCs were incubated for 3 min at room temperature in Krebs-Ringer bicarbonate (KRB) buffer containing 200 μM NBDG in the presence of indicated concentrations of D-glucose. Glucose concentrations were chosen according to previously published report (15, 29). Fluorescence intensity was randomly measured in regions of interest strictly limited to cumulus cell area. KRB was (in mM) 129 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2.0 CaCl2, 5.0 NaHCO3, and 10 HEPES, pH 7.4, supplemented with 0.1% bovine serum albumin.

Pharmacological inhibition of glucose transporters and gap junctions in COCs. The gap junction inhibitor carbenoxolone (CBX; Sigma) was initially solubilized in water to make a stock of 100 mM. The glucose transporter (GLUT) inhibitor cytochalasin B (CB; Sigma) was initially solubilized in 100% ethanol to make a stock of 100 mM. To check the effects of gap junction inhibition on glucose transport with glucose competition in COCs, COCs were preincubated for 30 min in M2 medium with and without 100 μM CBX and then moved to M2 medium containing NBDG with and without CBX for another 5-min culture at 37°C. To examine the role of GLUTs in glucose uptake of COC and DO, COCs and DOs were preincubated for 30 min in M2 medium with and without 100 μM CB and then added NBDG to M2 medium for another 5-min culture at 37°C. After three washes, cells were imaged for quantification of 6-NBDG uptake.

Generation of diabetic mice. To generate a type 1 diabetic model, female B6SJLJ1 mouse (Jackson Laboratories, Bar Harbor ME; 20–24 days old) received a single injection of streptozotocin at a dose of 190 mg/kg. Four days after injection, a tail blood sample was measured for glucose concentrations. If glucose levels were greater than 300 mg/dl, the animal was selected for use as a diabetic model. A few age-matched control mice were randomly selected.

Enzymatic measurement of free glucose in oocytes. To determine whether a high-glucose environment leads to free glucose accumulation in oocytes, two experimental models were employed. In the first, type 1 diabetic mice (see above) were used as an in vivo high-glucose environment model. Immature GV (germinal vesicles) and ovulated MII oocytes were collected from control and diabetic mice, and glucose levels were measured. In the second, an in vitro high-glucose environment was established by culturing COCs from normal mice in M2 medium containing 200 μM D-glucose for 60 min. This concentration was chosen because it is the upper range of blood glucose level in type 1 diabetic mice. In some experiments, to evaluate the role of glucose transporters and gap junctions under this condition, COCs were incubated in M2 medium containing 100 μM CB and/or CBX for 30 min prior to exposure to high-glucose medium; 50 μM L-glucose was included as an osmotic pressure control.

The detailed analytic procedure to measure free glucose has been described in Chi et al. (7, 33). Briefly, single oocytes freed of cumulus cells were frozen on a glass slide. After being freeze-dried overnight, samples were extracted in nanoliter volumes under oil. A glucose assay was designed to link reactions ending with NADP/NADPH, which were then enzymatically amplified in a cycling reaction, and a

byproduct (6-phosphogluconate) of the amplification step was measured in a fluorometric assay. Glucose levels are expressed as millimoles per kilogram wet weight (mM/Kw) based on the wet weight of 160 pg per oocyte. Absolute glucose concentrations can be calculated in picomoles by multiplying by 0.16.

Statistical analyses. Data are presented as means ± SE. Group differences were evaluated using Student’s t-test (GraphPad Prism 5, San Diego, CA). P < 0.05 was considered to be statistically significant.

RESULTS

Time course of 6-NBDG uptake in COCs. To measure glucose uptake, a variety of radiolabeled tracers have been used effectively. However, the spatial and temporal resolution of these methods is not high, and they require cell destruction. On the other hand, given that the intimate interconnection between cumulus cells and the oocyte could be critical for glucose uptake by the oocyte, it is important to include an examination of the live COC as a whole (as opposed to the isolated oocyte) (8). Hence, instead of using an isotope, we here employed a fluorescent glucose derivative (6-NBDG) to directly visualize glucose transport in live mouse COCs. The time-dependent uptake of 6-NBDG in COCs is shown in Fig. 1A. For both cumulus cells and oocytes, an initial rapid NBDG uptake was observed over the first 5 min, followed by a slower uptake phase that appeared to reach maximum accumulation after 15 min. Interestingly, we always found that the NBDG fluorescence in oocytes was apparently lower than that in surrounding cumulus cells at the 1-min incubation. With increasing culture time up to 3–5 min, similar fluorescence intensity was observed between them, evidenced by plot profiles (Fig. 1B; red arrows). This initial observation suggests that glucose probably was first taken up by cumulus cells and then progressed inward to the oocyte through gap junctional channels. The following experiments were designed to test this assumption.

Glucose transporters mediate 6-NBDG uptake by cumulus cells. To determine whether 6-NBDG uptake by cumulus cells is mediated via glucose-specific transport system and not via passive diffusion, we performed D-glucose competition assays. 6-NBDG fluorescence was measured in cumulus cells within live COCs in the absence or presence of various concentrations of D-glucose. As shown in Fig. 2A, 6-NBDG uptake was reduced by D-glucose in a dose response manner, inhibited by 34 ± 5.3 and 51 ± 6.8% in the presence of 25 and 50 mM D-glucose, respectively. To further confirm that 6-NBDG is transported to cumulus cells through GLUTs, we examined the effect of CB, an antagonist of GLUTs (17, 34, 52), on 6-NBDG uptake in live COCs. As shown in Fig. 2, B and C, the fluorescence intensity in cumulus cells was significantly decreased upon CB treatment (17.87 ± 2.52 vs. 52.99 ± 3.12 control, P < 0.05), suggesting GLUTs-mediated 6-NBDG uptake in cumulus cells. Meanwhile, CB treatment also resulted in reduced 6-NBDG fluorescence in the cumulus-enclosed oocytes (18.94 ± 2.56 vs. 44.15 ± 3.10 control, P < 0.05). This result indicates that 6-NBDG perhaps J) entered the oocyte via GLUTs directly or 2) moved from cumulus cells to the oocyte or J) that the two pathways worked in combination. To clarify this, cumulus cells were removed from COCs, and DO were treated with or without CB prior to 6-NBDG incubation. Significantly, 6-NBDG accumulated in denuded oocytes,
but GLUTs inhibition with CB was unable to block this entry (48.94 ± 3.28 vs. 45.95 ± 3.99 control, P > 0.05; Fig. 2, D and E). These data suggest that in mouse COCs glucose enters the oocyte largely through the connections between oocyte and cumulus cells not via resident oocyte GLUTs.

**Gap junctions are critical for 6-NBDG transport to oocytes.** Oocytes are coupled to the surrounding cumulus cells by gap junctions that permit the passage of small molecules between the two compartments. We reasoned that gap junctions might provide a route by which glucose enters oocytes. Therefore, we next examined the effect of gap junction inhibition on 6-NBDG trafficking in COCs. COCs were treated with or without CBX, a gap junction blocker (42), prior to NBDG exposure; representative images are shown in Fig. 3A. Compared with the control group, CBX markedly blocked 6-NBDG accumulation in the oocyte, within the COC (red arrows in insets), with no effect on cumulus cells. CBX is also known as a blocker of gap junction hemichannels (46) containing one connexon unit.

CBX treatment of denuded oocytes also reduced the 6-NBDG uptake (30.20 ± 2.54 vs. 41.43 ± 2.60 control, P < 0.05; Fig. 3B), suggesting the existence of functional gap junction hemichannels in denuded oocytes. Moreover, treating COCs with CBX and CB simultaneously abolished the 6-NBDG uptake of both oocyte and cumulus cells (oocyte: 14.32 ± 2.55 vs. 46.12 ± 4.2 control; cumulus: 12.22 ± 3.21 vs. 49.41 ± 4.72 control; P < 0.05; Fig. 3C). Collectively, these data suggest that glucose taken up by cumulus cells via GLUTs can be further transferred to oocytes through gap junctions.

**High-glucose environment induces glucose accumulation in oocytes.** To determine whether high-glucose conditions affect glucose levels in oocytes, we tested type 1 diabetic mice, which is an in vivo model of hyperglycemic milieu (blood glucose levels: 6.7 ± 0.8 mM control vs. 33.5 ± 10.7 mM diabetic). Immature GV and ovulated MII oocytes were isolated from control and diabetic mice, respectively, and then intracellular free glucose was measured enzymatically in individual
Notably, glucose content among oocytes from diabetic mice was consistently higher at both stages than that from control mice (GV: 2.67 ± 0.07 vs. 2.29 ± 0.02 control; MII: 1.07 ± 0.06 vs. 0.51 ± 0.02 control; \( P < 0.05 \); Fig. 4A). Moreover, in control oocytes, the glucose levels were reduced from GV to MII stage (2.29 ± 0.02 GV vs. 0.51 ± 0.02 MII), indicating that free glucose was metabolized during this transition. Second, we tested whether in vitro exposure of COCs to high glucose also increases glucose content in oocytes. COCs from normal mice were cultured in medium with different concentrations of \( \delta \)-glucose, and then single enclosed oocytes were obtained for glucose measurement. Consistent with the in vivo data, an approximately fourfold increase in glucose content was detected in oocytes exposed to 50 mM \( \delta \)-glucose compared with those exposed to 5 mM \( \delta \)-glucose (10.97 ± 1.36 vs. 2.66 ± 0.35, \( P < 0.05 \); Fig. 4B). Furthermore, disruption of gap junctions and/or GLUTs significantly blocked this enhancement; 50 mM \( \epsilon \)-glucose incubation was included as an osmotic control. Taken together, these results indicate that a high-glucose environment can increase glucose accumulation in oocytes.
oocytes through a GLUTs- and gap junctional channel-dependent mechanism.

DISCUSSION

In the present study, by imaging fluorescent glucose in live COCs, we present evidence for an intercellular pathway that transports glucose into oocytes: cumulus cells take up glucose via glucose transporters and glucose in cumulus cells can be further transferred into oocytes via gap junctions. Furthermore, we show that through this pathway a high-glucose environment induces elevated free glucose in the oocyte.

Glucose transport pathway in COCs. A long-established dogma of COC energy metabolism is that the oocyte is unable to use glucose as a sole energy substrate in the absence of cumulus cells and must be supplied with pyruvate (2). However, the presence of hexokinase (48), glucose-6-phosphate dehydrogenase (32), the pentose phosphate pathway (PPP) (16, 49), as well as GLUTs (36), in oocytes suggests a possible involvement of glucose metabolism in some oocyte functions. Remarkably, it is still controversial as to how and even whether glucose can enter oocytes. For example, some research groups have showed that denuded mouse and human oocytes can take...
Fig. 4. High-glucose environment increases glucose accumulation in oocytes. A: glucose levels were enzymatically measured in immature GV and ovulated metaphase II (MII) oocytes collected from control and diabetic mice, respectively. B: COCs were in vitro cultured in medium containing different concentrations of glucose with or without GLUT inhibitors and gap junctions, as described in MATERIALS AND METHODS. Glucose levels were measured in DOs following removal from the COC. Data are means ± SE (n = 30 COCs pooled from 3 replicates for each group). *P < 0.05.

Potential functions of free glucose in oocytes. We find that high-glucose exposure, both in vivo and in vitro, results in elevated glucose levels in oocytes within COCs through a GLUT- and gap junction-dependent mechanism (Fig. 4). Furthermore, we show that free glucose is metabolized in the transition from GV oocytes to the MII stage (Fig. 4A) for energy generation, biosynthesis, or conversion into a nonfree form (e.g., glycogen). These findings raise two important questions: what are the potential functions of the free glucose in the oocyte; and do high (or low) glucose levels impact on oocyte quality? Below, we speculate on some possibilities.

Emerging data suggest that glucose in the oocyte may be utilized primarily by the pentose shunt, which is more active than the glycolytic pathway and TCA cycle (3, 7, 47). Furthermore, use of PPP stimulators such as phenazine ethosulfate and pyrroline-5-carboxylate leads to a dose-dependent increase in germinal vesicle breakdown and glucose consumption in the mouse oocyte (14, 16). Similarly, manipulation of the PPP altered the developmental potential of porcine and bovine oocytes (10, 22). In particular, two metabolic intermediates of PPP, NADPH as a key regulator for glutathione reduction and phosphoribosyl pyrophosphate (PRPP) as a substrate for de novo purine synthesis, have been demonstrated to be important.

To clarify past findings, we used 6-NBDG to directly visualize glucose uptake in live COCs and employed inhibitors of GLUTs and gap junctions to examine the role of these two processes in glucose transport into the oocyte. First, our study provides direct evidence that fluorescent glucose can enter both cumulus-enclosed mouse oocytes and denuded oocytes (Fig. 2). A similar phenomenon was also reported in bovine COCs by Sutton-McDowell et al. (45). Second, we observed that inhibition of GLUTs leads to decreased glucose uptake in cumulus cells as well as in the enveloped oocyte (Fig. 2B). In support of these data, we have identified three GLUTs (GLUT1, -8, and -12) expressed in mouse cumulus cells by quantitative RT-PCR (36a). In addition, despite the presence of GLUT1, -7, and -9 in mouse oocyte (36), CB treatment has no apparent effect on 6-NBDG incorporation to denuded oocytes (Fig. 2D), indicating that the GLUT system may play only a minor role in oocyte glucose uptake. Compared with the radiolabeled glucose tracers, the 6-NBDG method has better spatial and temporal resolution but is less quantitative and sensitive. Hence, on the basis of this study we cannot completely rule out the possibility that some glucose directly enters the oocyte via GLUTs. Third, we show experimentally for the first time that disruption of gap junctions between oocyte and cumulus cells dramatically blocked glucose transport into the oocyte (Fig. 3). Gap junctions are specialized structures occurring at points of very close cell-cell contact, directly connecting the cytoplasm of two cells, allowing various molecules and ions to pass freely between the cells. One gap junction channel is composed of two connexons (or hemichannels), which connect across the intercellular space (31). Interestingly, CBX as a blocker of hemichannels (25) reduces the 6-NBDG uptake in denuded oocytes (Fig. 3B), implying that the connexons in denuded mouse oocytes are partly functional. It should be pointed out that simple 6-NBDG diffusion into oocytes perhaps occurred, because the weak fluorescence can still be detected in COCs treated with both inhibitors (Figs. 2 and 3). Figure 5 presents a model describing the pathway for intercellular transport of glucose into the oocyte, where glucose is taken up by cumulus cells via GLUTs and then transported into the oocyte through gap junctions.

GLUCOSE TRANSPORT IN CUMULUS-OOCYTE COMPLEX

Fig. 5. Model for the intercellular glucose transport pathway in mouse COC. Schematic diagram illustrating that glucose is taken up by cumulus cells via GLUTs and then transported into the oocyte through gap junctions. See text for details.
for meiotic regulation within oocytes (14, 45). These data indicate that glucose participates in modulating oocyte maturation via the PPP. In addition to being an energy source, glucose also has important regulatory functions, controlling stress resistance, growth, and development in bacteria, yeasts, plants, and animals (38). Several glucose-sensing pathways have been well documented in yeast. One of them employs the cyclic AMP (cAMP) as a second messenger to regulate cell signaling (26). It is interesting to note that cAMP plays a crucial role in meiotic resumption of the oocyte (11). Therefore, free glucose in oocytes may exert effects on oocyte maturation by cAMP pathway.

Both the in vivo hyperglycemic environment and in vitro high-glucose culture have been reported to adversely affect the developmental competence of oocytes (9, 15, 21, 28, 51). Glucose accumulation in oocytes perhaps partly mediates such effects. For instance, mitochondrial dysfunction resulting from high glucose has been reported in various cell types (40, 53). Low glucose is necessary to protect the oocyte against oxidative stress under high-oxygen conditions (21). High glucose may inhibit enzymes responsible for GSH synthesis, thus impairing the oocyte’s ability to reduce reactive oxygen species (ROS) (28). Consistent with this notion, altered mitochondrial reactive oxygen species and glutathione contents. Exp Zool 198: 375–382, 1976.


