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EXPRESSION AND POSSIBLE FUNCTION OF GLUCOSE TRANSPORTER PROTEIN GLUT1 DURING PREIMPLANTATION MOUSE DEVELOPMENT FROM OOCYTES TO BLASTOCYSTS

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SUMMARY: A micro-Western analysis method both sensitive and quantitative enough to analyze oocytes and embryos is developed. GLUT1 protein is present in mouse oocytes and preimplantation embryos and levels are increased by fertilization and with time in ensuing embryonic development; the levels were 20-fold greater in blastocysts than in unfertilized oocytes. Similar increases were observed in glucose uptake by oocytes and embryos, suggesting that they may depend on GLUT1 expression. These results suggest that GLUT1 expression may explain a switch in substrate preference of the embryo from pyruvate to glucose during preimplantation development.

Glucose is unable to support the development of oocytes and early embryos until the 4-cell stage (1). There is a characteristic switch in the substrate preference of embryos from pyruvate, during the early cleavage stages, to glucose, after the 8-cell stage (2,3). Glucose becomes the predominant energy source at the blastocyst stage. This change in early embryogenesis may depend upon the ability of oocytes and embryos to take up glucose (3-5). Thus, several investigators have attempted to assess the viability of embryos by measuring glucose uptake (3,6).

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ABBREVIATIONS: GLUT, glucose transporter; 2-DG, 2-deoxy-D-glucose; mBWW, modified Biggers, Whitten and Whittingham medium; hCG, human chorionic gonadotropin; SDS, sodium dodecyl sulfate.

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Glucose can be transported across membranes by two different mechanisms: a sodium-coupled active carrier system, or a sodium-independent facilitative glucose transporter (GLUT) system (7). It has been demonstrated that glucose uptake by preimplantation embryos is mediated by the latter system (8). Recently, mRNA's of some GLUT genes have been observed by the reverse transcription and polymerase chain reaction technique and their product proteins have been histochemically demonstrated in early mouse embryos (9). Thus, it may be possible that the glucose transporter protein is expressed and may play a role in early embryogenesis. Understanding the characteristics of glucose transporter protein and its changes during development may be important, especially for advances in *in vitro* fertilization techniques in man.

We have developed a micro-Western blot technique sensitive enough to determine not only the presence of, but also the quantities of the GLUT protein in a relatively small number of oocytes and embryos. We have also quantified the uptake of glucose by the embryos to assess a possible function of the glucose transporter protein. This report describes the developmental changes of the glucose transporter isoform GLUT1 expressed in mouse oocytes and embryos during the preimplantation period.

MATERIALS AND METHODS

Embryos: B6C3F1 female mice were superovulated with 5 IU of pregnant mare serum gonadotropin, followed 48 hours later by 5 IU of hCG. Mating with males of the same strain was confirmed by the presence of a vaginal plug. Fertilized oocytes, 2-cell embryos, 8-cell embryos, and blastocysts were obtained at 16, 40, 64 and 88 hours after hCG administration, respectively, by flushing the oviducts or the uterii. Unfertilized oocytes were also collected 16 hours after hCG injection, with a brief treatment with hyarulonidase to remove cumulus cells.

Solubilization of samples: Eighty to 500 oocytes and embryos were collected in group in about 1.5 μl droplets of phosphate buffered saline (pH 7.4), containing 3 mg/ml of polyvinylpyrroridone, placed at the bottom of a small well (4x3 mm) filled with paraffin oil to prevent evaporation. They were solubilized by the addition of the 1.5 μl of lysis buffer composed of 10% SDS; 1mM phenylmethylsulfonyl fluoride; 125 mM dithiothreitol; 20% glycerol; 0.002% bromophenol blue (BPB); and 125 mM Tris-HCl (pH 6.8). They were heated at 60 ℃ for 6 min to avoid aggregation of glucose transporter proteins and stored at -80 ℃ under mineral oil. As standards for GLUT1 and GLUT2 Western blotting, human erythrocyte ghosts (10) and mouse liver membrane fractions (11) were prepared. After determination of protein content in the ghosts and the membrane by the method of Lowry *et al.* (12), they were solubilized in the lysis buffer and stored at -80℃ as described above.

Micro-Western blot analysis: Lysed samples and standards were heated again under mineral oil at 60℃ for 6 min immediately before being loaded onto slab gels. Sodium dodecyl sulfate-polyacryamide gel electrophoresis was performed according to the method of Laemmli (13) using a microslab gel (38x38x1 mm, 14) with slight modification. Briefly, to concentrate the protein bands separated, the well size was reduced to 1 x 1 x 8 mm for 3 μl of sample volume. Samples, standards, and prestained molecular weight markers (Bio-Rad, Richmond) were run through the stacking gel and the separating gel at 5 and 10 mA/gel, respectively. The electrophoresis was stopped when the BPB line traveled 10 mm downwards in the separating gel. The proteins were then electrophoretically transferred to nitrocellulose membranes in the transfer buffer (0.025 M Tris-base, 0.19 M glycine and 20 % methanol) containing 0.01 % SDS to improve the transfer efficiency.

The membranes, after electrophoretic transfer, were blocked at 4 ℃ overnight in a solution containing 3% fraction V bovine serum albumin in TBS (150 mM NaCl, 0.1 % NaN_3 and 10 mM Tris-HCl, pH7.4), and incubated with an affinity purified antibody against the C-terminal domain of human GLUT1 (11) or mouse GLUT2 (15) for 2 hours at room temperature at 5 μg/ml in TBS containing 2.5% nonfat dry milk. The membranes were washed three times at room temperature for 20 min each time in the rinse buffer (1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.1% Triton X-100) and then reblocked in TBS with 2.5% nonfat dry milk. The blocked filters were incubated at 37℃ for 90 min with 5-9 x 10^5 cpm/ml of ^{125}I-labeled protein A (ICN Biomedicals, California) in TBS containing 2.5% nonfat dry milk, according to the method of Burnette (16). The membranes were then washed three times as above and the radioactivities were determined with Fujix BAS 2000 Bio-Image Analyzer (Fuji Photo Film Co., Ltd., Kanagawa, Japan) after exposure for 24-48 hours.

Glucose uptake: Five oocytes and individual embryos were incubated in 4 μl of mBWW medium (17) containing 25 μM [^{3}H]-2-deoxy-D-glucose (2-DG, Amersham, Littel Chalfont Buckinghamshire, 17Ci/mmol) instead of glucose. They were incubated for 60 min at 37℃ under atmosphere of 95% air and 5% CO_2 with 100% moisture. Oocytes and embryos were then washed five times by transferring each time to 100 μl of mBWW medium free of glucose. The uptake of 2DG into five oocytes or each embryo was counted with 1 ml of Aquasol solution in a Beckman scintillation counter.

RESULTS

Immunoreactive GLUT1 was present in oocytes and embryos as shown in Fig. 1. There was a broad band of reactivity on Western blotting with a mean molecular weight of approximately 50,000 daltons. However, GLUT2 was not clearly detectable in 500 blastocysts when the immunoreactivity was found with 25 ng of protein on the micro-Western blotting for hepatocyte membrane fractions (data not shown). The intensity of GLUT1 immunoreactivity significantly increased on fertilization (Fig. 1A) and as the embryo developed (Fig. 1B). The standard curve is linear in a wide range from 0.4 to 6 ng of human erythrocyte ghost protein

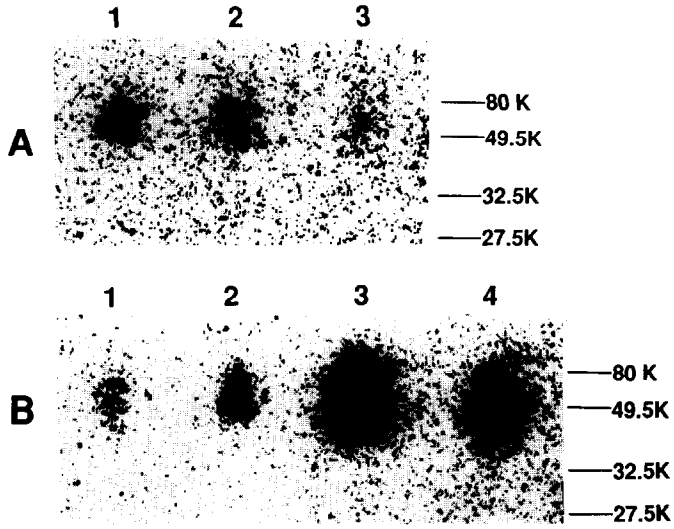

Fig.1. A representative micro-Western blot patterns of GLUT 1 protein in mouse oocytes and embryos. The numbers on the right-hand scale indicate the position of the molecular weight markers in kDa. Strips of separating gels approximately 10 mm width were cut between the range of molecular weights as shown in the Figure and the proteins were transferred to a nitrocellulose membrane. A: lane 1, 2 ng of erythrocyte ghost protein; lane 2, 500 unfertilized oocytes; lane 3, 500 fertilized oocytes. B: lane 1, 200 2-cell embryos; lane 2, 200 8-cell embryos; lane 3, 200 blastocysts; lane 4, 2ng of ghost protein.

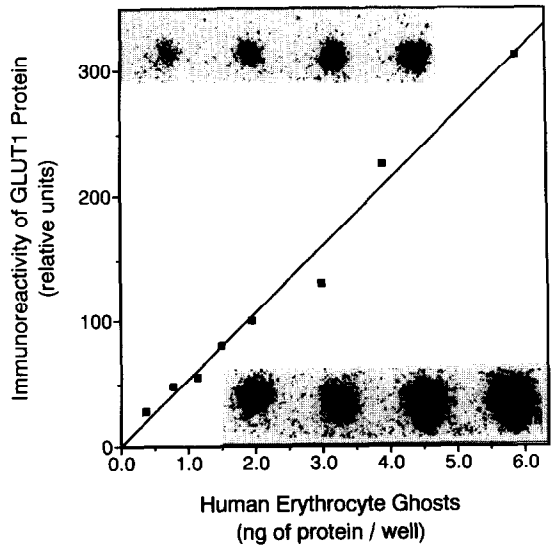

Fig.2. Standard curve of GLUT 1 protein using human erythrocyte ghosts. The immunoreactivities relative to that observed in 2 ng of ghost protein (100 units) are plotted on the ordinate. The immunoreactive bands in the upper row correspond to 4 plots from 0.4 to 1.6 ng of protein and those in the lower row from 2.0 to 6.0 ng in the increasing order. The background was 12 units.

Table 1. Glucose transporter protein (GLUT1) in mouse oocytes and embryos

Samples	Hours after hCG injection	Number of experiments	Number of samples analyzed	GLUT1 immunoreactivity
unfertilized oocytes	16	3	500	21.5 ± 0.7
fertilized oocytes	16	3	500	28.8 ± 1.5*
2-cell embryos	40	3	200-500	37.6 ± 6.4**
8-cell embryos	64	3	200-350	47.2 ± 4.5**
blastocysts	88	4	80-200	445.8 ±20.3**, §

GLUT1 protein immunoreactivity was determined by micro-Western blotting. ^{125}I-protein A binding to GLUT1 protein per 250 samples was expressed in percent relative to the standard (2 ng of protein of human erythrocyte ghosts). The results presented are the mean ± SEM.
* and **, $p<0.05$ and $p<0.01$ compared with unfertilized oocytes, respectively.
§, $p<0.01$ compared with that obtained 24 hours earlier.

(Fig. 2). GLUT1 immunoreactivity levels in oocytes and embryos were determined in this liner range and the results were normalized and expressed per 250 oocytes or embryos (Table 1). The GLUT1 levels, examined every 24 hours after fertilization, were increased by fertilization and embryonic development. The most remarkable increase was observed between the 8-cell and the blastocyst stages, which was approximately 10-fold.

2-DG incorporation into the fertilized oocyte was 3.29 ± 0.09 fmol/oocyte/hour, which was significantly greater than that of the unfertilized oocyte (1.22 ± 0.88) ($P<0.01$). 2-DG uptake increased exponentially between the 2-cell embryo and blastocyst stages (Fig. 3) There was a greater than 300-fold increase in 2-DG uptake in the blastocyst (396 ± 26 fmol/embryo/hour) as compared with the unfertilized oocytes.

DISCUSSION

The present micro-Western blot made it possible to analyze a small amount of samples (ex., 80 blastocysts) with appropriate accuracy and reproducibility, and revealed that GLUT1 protein is expressed in the oocytes and preimplantation embryos (Fig. 1). There have been several lines of evidence that glucose is transported by the GLUT system in embryos (8), and GLUT1 and 2 are possible

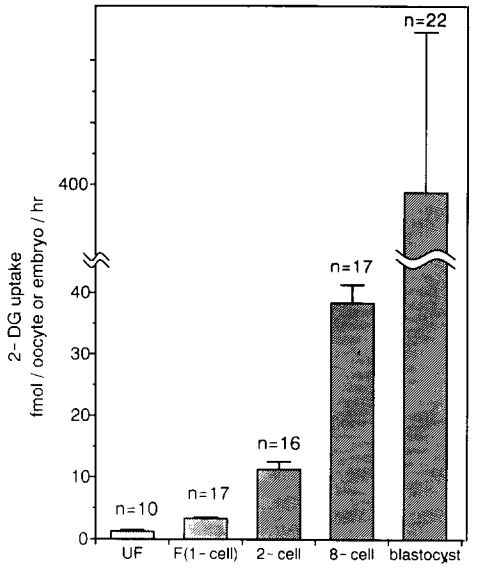

Fig 3. 2-Deoxyglucose uptake by the mouse oocytes and embryos.
The T on the top of the bar indicates SEM. The differences between any pair of developmental stages are significant ($P<0.01$). n=number of determinations. UF, unfertilized oocyte; F(1-cell), fertilized oocyte.

candidates involved in this system, because their mRNA's and immunohistochemical reactions have been demonstrated (9). Based on our present data that GLUT1, but not GLUT2, is detectable, it may be GLUT1 which is important for glucose uptake by oocytes and embryos during the preimplantation period.

We have also shown that levels of GLUT1 increase when oocytes are fertilized or embryos developed (Table 1). Similarly, significant increases are observed in the glucose uptake by the oocytes and embryos (Fig. 3), which is compatible with a previous report (3). These results give some insight into the characteristic switch in the substrate preference of embryos from pyruvate to glucose. Concomitant increases in glucose uptake and GLUT1 expression strongly suggest that elevated levels of GLUT1 are responsible for the increased capacity of glucose uptake, which may be at least in part related to substrate preference. However, it is also plausible that levels of enzymes of glucose metabolism may regulate the main energy source of the oocytes and embryos, because low hexokinase activity has been reported to explain the inability of mouse, rat, and human oocytes and embryos to utilize glucose (18-21). It is interesting to note that

hexokinase activity in the blastocyst is markedly increased compared with those of oocytes and early embryos (18, 20).

It must be noted that the rates of increase in GLUT1 and glucose uptake were not necessarily parallel; the increase in GLUT1 was 20-fold while that of glucose uptake was more than 300-fold during the experimental period. One plausible explanation is that levels of the glucose transporter described here includes both membrane and cytoplasmic protein as the preparation is done without membrane separation. Further study may elucidate a possible preferential localization of glucose transporter from cytoplasm to plasma membrane during development. Another possible explanation is that part of GLUT1 on the plasma membrane does not function in the early stages of embryonic development.

In summary, our results indicate that GLUT1 expressions and glucose uptake of embryos increase during preimplantation development. They may be important for analyzing biochemically the process of preimplantation development, because morphological criteria, such as the rate of blastocyst formation *in vitro*, are not so quantitative and have limited usefulness. Moreover, these changes are observed when the embryos develop *in vitro* although the increases *in vitro* are significantly smaller than those *in vivo* (our unpublished data). Thus, determination of GLUT1 or glucose uptake may serve as a tool to investigate not only developmental changes, but also a possible involvement of certain factors influencing embryonic development. In fact, studies examining roles of growth factors, such as epidermal growth factor, are now in progress in our laboratory using the methodology described here.

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