Glucose metabolism in the trophectoderm and inner cell mass of the rabbit embryo

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Summary. The metabolism of glucose in the intact Day-6 and -7 post coitum (p.c.) rabbit blastocyst and in the separated trophectoderm and inner cell mass (ICM) of the Day-7 p.c. embryo was investigated. At Day-6 p.c., glucose traversed the trophectoderm with a half-time of 39 ± 9.3 min, and was metabolized to CO₂ at a rate of 25.5 ± 1.6 nmol·cm⁻²·h⁻¹. Neither the Na⁺ ionophore, amphotericin B, nor cyclic AMP had an effect on glucose metabolism to CO₂. Lactate production by the Day-6 blastocyst was largely independent of glucose. At Day-7 p.c. in the intact embryo, CO₂ production from glucose significantly decreased to 7.76 ± 2.8 nmol·cm⁻²·h⁻¹. Per unit surface area, the metabolism of glucose to CO₂ was similar in the separated Day-7 p.c. trophectoderm and ICM. We conclude that the rabbit blastocyst is not highly dependent on glucose, and that the ICM does not utilize glucose as a metabolite to a greater extent than does the trophectoderm, at least in the Day-7 p.c. embryo.

Keywords: rabbit; blastocyst; glucose; carbon dioxide; inner cell mass

Introduction

The preimplantation rabbit embryo relies on a number of different mechanisms to provide metabolic energy. Up to the late morula stage, the embryo metabolizes glucose by the hexose monophosphate shunt pathway (Fridhandler, 1961). Later in development, at the stage when formation of the blastocoele occurs, the preferential metabolic pathway for glucose oxidation becomes glycolysis and the tricarboxylic acid cycle (Fridhandler, 1961). However, in the rabbit blastocyst pyruvate is converted to CO₂ at a rate greater than glucose (Brinster, 1969), indicating that metabolites other than glucose can be utilized by the embryo. In addition, it has also been shown that the blastocyst will increase O₂ consumption in response to an energy demanding load (Benos & Balaban, 1980).

Despite these studies, the nature and localization of these metabolic pathways are not clearly understood. At this time, it is not clear what substrate supports metabolism during the expansion of the blastocoele, a period during which metabolic demands will be high as a result of the vectorial transport of ions. Whether the inner cell mass (ICM) and the trophectoderm metabolize glucose differently has also not been assessed.

Robinson et al. (1990), demonstrated that a glucose analogue, 3-O-methyl glucose, traversed the trophectoderm of the rabbit blastocyst. It was additionally shown in the same study that this transport was accomplished by Na⁺-independent transport proteins localized to both the apical and basolateral membranes. The physiological function of this transport system during the preimplantation developmental period of the rabbit embryo is unknown. At least two possibilities exist. First, these glucose transport proteins may act as water channels, a function first proposed by

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Fischbarg et al. (1987). The rabbit blastocyst triples its volume between Day 6 and 7 post coitum (Biggers et al., 1988). Water channels would facilitate this expansion process. Second, the presence of glucose transporters in the trophodermal cells enables glucose to enter the blastocoelic cavity and may thus provide the ICM with this metabolic substrate.

If the second hypothesis is true, then glucose will traverse the trophoderm without being degraded, and the ICM will use glucose as a metabolic substrate. We tested this hypothesis by examining the transport of glucose across the trophoderm and its metabolism to CO₂. In addition, we examined the CO₂ generation from glucose in both the separated trophoderm and ICM.

Materials and Methods

**Blastocyst collection.** New Zealand White does (6-9 months of age) were double mated to fertile bucks. At 6 or 7 days post coitum (p.c.), the female rabbits were killed by i.v. injections of 1·5 ml T-61 euthanasia solution (American Hoechst Corp., Somerville, NJ, USA). The embryos were collected as previously described (Robinson et al., 1990) into Krebs'–Ringer–bicarbonate with glucose (KRBG) consisting of (in mM): NaCl, 119; NaHCO₃, 25; KCl, 4·7; KH₂PO₄, 1·2; MgSO₄, 1·2; CaCl₂, 1·7; d-glucose, 5·5; and N₂-hydroxethylpiperizine-N'₂-ethanesulphonic acid (Hepes), 20; pH adjusted to 7·4. ‘Modified KRBG’ was identical to KRBG except that the glucose concentration was 1 mM, and Hepes was omitted.

**Influx measurements.** Day-6 p.c. blastocysts were incubated in modified KRBG with 5 µCi [U-¹⁴C]glucose/ml for predetermined times. After this incubation, the blastocysts were washed 3 times with modified KRBG at 4°C, measured and punctured with a glass pipette (tip = 5 µm), and the blastocoelic fluid was withdrawn. No appreciable washout has been observed using this method (Robinson et al., 1990). Two 10-µl samples of the incubation fluid were taken to calculate specific activity. Up to 10 µl blastocoelic fluid were counted for radioactivity. Samples were counted by adding 4 ml Aqueous Counting Scintillant (Amersham Corp., Arlington Heights, IL, USA), and then assaying for radioactivity with a Packard scintillation counter Model 2000CA.

**Carbon dioxide production.** Blastocysts were rinsed in filter sterilized and gassed (95% O₂, 5% CO₂) modified KRBG before the start of the experiment. Individual Day-6 and -7 p.c. embryos were measured as previously described (Robinson et al., 1990) and incubated in the sterile modified KRBG at 37°C in a 12 × 75 mm glass test tube which was placed in a glass container with a rubber diaphragm top. Day-6 and -7 p.c. embryos were incubated in 250 µl and 500 µl of the buffer, respectively. Amphotericin B, phloretin, chlorophenylthio cyclic AMP, and dideoxyforskolin were added at this time. The time of preincubation was 1 min. An empty 10 × 75 mm glass test tube was also placed in the glass container. This tube served as the CO₂ trap. The experiment was initiated by pipetting [U-¹⁴C]glucose (sp. act. 265 mCi/mmol; New England Nuclear) into the test tube containing the embryo, to a final concentration of 10 µCi/ml. The experiment was terminated by adding 1 n H₂SO₄ at a volume twice that of the incubation solution through the diaphragm with a syringe, and then adding 0·5 ml 2·5 n-NaOH to the empty test tube. After standing at room temperature overnight, the 2·5 n-NaOH was decanted into a scintillation vial, neutralized with 1 ml 1 n-Tris–Cl, and after the addition of 20 ml scintillation fluid, counted as described above.

For the separated trophoderm/ICM experiments, after the blastoderm dimensions were measured, the ICM and trophoderm were mechanically separated. The trophoderm and ICM from a single blastocyst were individually incubated in 100 µl modified KRBG.

**Lactate measurements.** Day-6 p.c. blastocysts were incubated in 250 µl modified KRBG unless otherwise specified. The experiments were terminated by withdrawing 200 µl of the incubation medium and assaying for lactate with a kit from Sigma Chemical Co., St. Louis, MO, USA.

**Chemicals.** Unless otherwise stated, the chemicals used were reagent grade and were purchased from Sigma Chemical Co.

**Data analysis.** Data are expressed as mean ± standard error. The statistical test used to analyse for significance in each case is given in the figure legend.

Results

Previous results indicated that a non-metabolizable analogue of glucose, 3-O-methyl glucose (3-OMG) enters the blastocoelic with a characteristic half-time, and equilibrates with the 3-OMG concentration in the outer fluid (Robinson et al., 1990). To determine whether this was also true for
metabolically active glucose, we examined the influx of 1 mM-glucose across the trophectoderm of the Day-6 p.c. rabbit embryo (Fig. 1). Glucose entered the blastocoele with a time course similar to that of 3-O-methyl glucose (Robinson et al., 1990). The half-time of uptake was 39 ± 9.3 min, and the maximum uptake was 63.6 ± 0.9 nmol·cm⁻². The calculated maximum uptake corresponded to a blastocoele concentration of 1 mM-glucose. The similar half-time of glucose and 3-OMG uptake suggests that there was not a high rate of glucose metabolism in the trophectoderm.

![Graph depicting the time-course of transtrophectodermal glucose transport in Day-6 p.c. rabbit blastocysts.](image1)

**Fig. 1.** Graph depicting the time-course of transtrophectodermal glucose transport in Day-6 p.c. rabbit blastocysts. The curve was fitted with an iterative non-linear curve-fitting programme. The half-time of uptake is 39 ± 9.3 min, and the maximum uptake is 63.6 ± 0.9 nmol·cm⁻². When not shown, the error bars are within the circles (n = 3 for each point).

We next examined the CO₂ production from glucose in Day-6 p.c. blastocysts to determine the rate at which the blastocyst uses glucose as a metabolic substrate. After a slight delay (6.7 min), CO₂ production was linear for up to 60 min. Phloretin at 10⁻⁴ M, which inhibits uptake of hexoses across the trophectoderm (Robinson et al., 1990), significantly (P < 0.05) decreased the CO₂ production.

![Graph depicting the time-course of CO₂ production from glucose in the Day-6 p.c. rabbit embryo.](image2)

**Fig. 2.** Graph depicting the time-course of CO₂ production from glucose in the Day-6 p.c. rabbit embryo. The CO₂ production from embryos treated with 5 μg amphotericin B/ml (open triangles) was not significantly different from the control (closed circles). Treatment with 10⁻⁴ M-phloretin (open circles) significantly decreased the slope (analysis of covariance, P < 0.001), (n = 3–5 for each point).
production (Fig. 2), thus indicating that cellular mechanisms are responsible for the degradation of glucose to CO₂.

The sodium ionophore amphotericin B, at 5 µg/ml, increased the active transport of sodium into the blastocoel of the Day-6 p.c. rabbit blastocyst, which in turn increased O₂ consumption in the blastocyst (Benos & Balaban, 1983). Amphotericin B at this same concentration had no effect on the evolution of CO₂ from the Day-6 p.c. embryo (Fig. 2).

Cyclic AMP decreased the active transport of Na⁺ and Cl⁻ in the blastocyst (Benos & Biggers, 1983). To determine whether a decrease in ion transport affected glucose metabolism to CO₂, we tested the effect of chlorophenylthio cyclic AMP (CPT cAMP; 10⁻⁴ M) on CO₂ production. CPT cAMP had no effect on CO₂ production (control, 25·4 ± 1·3 nmol·cm⁻²·h⁻¹ (n = 4); 10⁻⁴ M-CPT cAMP, 25·5 ± 1·6 nmol·cm⁻²·h⁻¹; t test, n = 5).

The dependence of lactate production on available glucose in Day-6 p.c. blastocysts was next examined. Blastocysts were incubated in modified KRBG and in KRB with 1 mM-3-O-methyl glucose (3-OMG) in place of glucose. Lactate evolution was linear over 60 min (Fig. 3), and was not highly dependent on external glucose. The difference in rate (i.e. with and without glucose) was not significant (control, 31·7 ± 4·7 nmol·cm⁻²·h⁻¹; 3-OMG, 17·6 ± 6·8 nmol·cm⁻²·h⁻¹; t test, 0·10 > P > 0·05). Initially, we used dideoxyforskolin (ddF; 10⁻⁴ M) to inhibit uptake of glucose into the trophectoderm (Robinson et al., 1990), because phloretin caused an increase in the optical density in blank tubes in the colorimetric lactate assay. Surprisingly, lactate production significantly (t test; P < 0·05) increased from 35·7 ± 4·0 nmol·cm⁻²·h⁻¹ in the control situation, to 58·2 ± 4·6 nmol·cm⁻²·h⁻¹ when exposed to ddF. Because ddF does effectively block glucose transport in the trophectoderm (Robinson et al., 1990), this result, along with the independence of lactate production on glucose, indicates that the formation of lactate is not closely related to the availability of exogenous glucose.

![Graph depicting the production of total lactate from Day-6 p.c. rabbit blastocysts.](image)

**Fig. 3.** Graph depicting the production of total lactate from Day-6 p.c. rabbit blastocysts. The slope of the control embryos (circles) is not significantly different from the experimental condition when the glucose is replaced with 3-O-methyl glucose (closed squares; t test), (n = 4–5 for each point).

We next tested whether there was a correlation between developmental age and the utilization of glucose. CO₂ generation in the Day-7 p.c. blastocyst was significantly lower when compared with the Day-6 p.c. embryo (7·76 ± 2·8 nmol·cm⁻²·h⁻¹ at Day 7 p.c., n = 4; 22·2 ± 2·2 nmol·cm⁻²·h⁻¹ at Day 6 p.c., n = 3; t test, P < 0·05).

The lack of correlation between trophectodermal metabolism and CO₂ evolution suggested that the ICM may be the primary producer of CO₂ from glucose, and the trophectoderm is largely
glucose-independent. We tested this hypothesis by incubating separated trophoderm and ICM from Day-7 p.c. embryos and comparing CO₂ production in these separate tissues. The combined CO₂ production from ICM and trophoderm of the same blastocyst was not significantly different from that of the control intact embryos (Table 1; ANOVA, P > 0.05). It appears that the trophectoderm alone is responsible for the production of CO₂ by the embryo.

| Table 1. CO₂ production from intact and separated Day-7 p.c. rabbit blastocysts |
|--------------------------------------|-----|-----|
| Condition | CO₂ (nmol·cm⁻²·h⁻¹) | No. |
| Intact    | 7.8 ± 2.8          | 4   |
| ICM       | 0.7 ± 0.6          | 5   |
| Trophoderm| 10.6 ± 2.4         | 5   |
| ICM + trophoderm | 11.4 ± 2.2 | 5   |

**Discussion**

In a comprehensive study of glucose metabolism in the rabbit embryo, Fridhandler (1961) demonstrated that glucose oxidation occurs via the hexose monophosphate shunt pathway before blastulation, after which glycolysis and the tricarboxylic acid (TCA) cycle predominate. Our results, when examined in conjunction with those of a previous study (Benos & Balaban, 1980), reveal that glucose oxidation, although it occurs at a significant rate, is most probably not the major pathway by which the rabbit blastocyst obtains its energy. At Day-6 p.c., the ratio of glucose oxidation, as measured by CO₂ production to O₂ consumed, is 0.2, and at Day-7 p.c. this ratio is decreased to 0.065. The lowest possible ratio of total CO₂ produced to O₂ consumed, which would indicate only fat metabolism, is 0.7. Because the calculated ratios for glucose oxidation to O₂ consumed in this study are far lower than 0.7, clearly another metabolite is being used as the major energy substrate.

Brinster (1969) demonstrated that rabbit embryos at Day-6 p.c. produce CO₂ from pyruvate to a much greater extent than from glucose. This finding suggests that the TCA cycle is operative at this stage of development and that, under our conditions, pyruvate derived from glucose is not the preferred metabolic substrate. Thus, another metabolite is being preferentially shunted to the TCA cycle.

Possible candidates for a metabolic substrate in the rabbit embryo are the neutral glycerides (mono, di, and triacylglycerols). This would not only explain the difference between the CO₂ produced from glucose and the theoretical minimum CO₂ production, but also the lack of a strong dependence of lactate production on the presence of glucose. The glycerol could be converted to lactate, and the fatty acids would be oxidized via β oxidation pathways. In fact, it has been shown that the mouse embryo has a large neutral glyceride pool and can utilize fatty acids for metabolism via β oxidation (Flynn & Hillman, 1980).

Glucose metabolism is also independent of increases in O₂ respiration. A previous report (Benos & Balaban, 1983) demonstrated that the Na⁺ ionophore, amphotericin B at 5 μg/ml, caused an increase in both aerobic respiration and glycolysis (as judged by lactate production) resulting from the increase in vectorial Na⁺ transport. Our current findings indicated that this increase in metabolism was not associated with an increase in glucose oxidation. We speculate that neutral glyceride pools are mobilized when there is an additional load to normal energy requirements.

On a per cell (or cm²) basis, the utilization of glucose decreases between Day 6 and 7 p.c.. Benos & Balaban (1983) found that there was no change in oxygen consumption per cm² during this...
period. This decrease in glucose utilization must be due to intracellular mechanisms and is not a result of glucose entry, because 6- and 7-Day p.c. blastocysts display similar hexose transport characteristics (Robinson et al., 1990).

The present study also suggests that the ICM may also be functioning in primarily a glucose-independent fashion because the rate of glucose oxidation in the ICM was similar to that in the trophectoderm as a whole. At Day 7 p.c., the surface area of the ICM is 5–10% that of the trophectoderm (unpublished observation). Thus, on a per cm$^2$ basis, the CO$_2$ generated from glucose is 7–14 nmol·cm$^{-2}$·h$^{-1}$, which is similar to the value found in the trophectoderm.

Total lactate production in the presence and absence of glucose has been previously examined (Mounib & Chang, 1965). These investigators found that lactate could be generated in the absence of glucose, and when 0.93 mm-glucose was added, the rate of lactate production increased 2.5-fold. This is in close agreement with our data which indicate that there is a 1.8-fold increase in the rate of lactate production in the presence of glucose when compared to the rate in the absence of glucose. The surprising finding was that lactate production increased when the embryos were exposed to dideoxyforskolin. Obviously, dideoxyforskolin has some effect other than blocking glucose transport, because replacement of glucose with 3-OMG would have had a similar effect if the increase in lactate production was a result of glucose deprivation. This effect of dideoxyforskolin on lactate production could be occurring at any part of the triacylglyceride metabolic pathways if in fact, the embryo is dependent on these pathways. Alternatively, dideoxyforskolin could be influencing lactate production indirectly by interfering with another aspect of cellular function. For example, dideoxyforskolin has been observed to block voltage-dependent K$^+$ channels directly (Hoshi et al., 1988). These non-cAMP mediated effects of forskolin and its analogues may be related to the compound interacting with hydrophobic sites because water-soluble forskolin analogues do not have the same effect (Wagoner & Pallotta, 1988).

Quinn & Wales (1973) found that exogenous glucose was incorporated into the acid soluble fraction in the Day-6 p.c. rabbit blastocyst at a rate 4.9 times greater than into the protein fraction, and 37 times greater than into the lipid fraction. Of this acid-soluble fraction, a substantial portion was incorporated into neutral compounds. The authors suggested that this fraction represented glucose or glycogen. In addition, it was also found (Quinn & Wales, 1973) that, of the total glucose carbon in the blastocoel and embryonic tissues, about 20% was incorporated into the embryonic tissue as acid-soluble compounds, proteins, or lipids. These observations, when combined with the low rate of glucose catabolism, indicate that glucose may be more important as an anabolic compound rather than as a catabolite.

Glucose metabolism in mouse and rabbit embryos has been extensively examined (Fridhandler, 1961; Mounib & Chang, 1965; Brinster, 1967, 1969; Quinn & Wales, 1973; Flynn & Hillman, 1978; Khurana & Wales, 1987a, b). Our current findings suggest that the rabbit embryo is not highly dependent on glucose metabolism and may be more dependent on neutral glyceride pools. Our findings also indicate that transtrophectodermal transport of glucose may not be an absolute requirement for viability. We are then left to consider other reasons for the existence of this glucose transport system. Perhaps glucose is required by the cell for purposes other than an energy-producing metabolite. For example, glucose may be required to form the glycerol portion of the neutral glycerides, as was proposed for the mouse embryo (Flynn & Hillman, 1978) or, conversely, is converted to glycogen to be used later as an energy source (Quinn & Wales, 1973).

Animals used in this study were maintained in accordance with the guidelines of the Animal Use Subcommittee at the University of Alabama at Birmingham and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW publication No. (NIH) 85-23, revised 1985].

We thank Dr Claire Chatot for helpful suggestions.

This work was supported by NIH grants HD21302, and HD07069.
References


Received 25 May 1990