Carbohydrate metabolism in ‘delayed implanting’ mouse blastocysts undergoing activation in utero and in vitro

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Summary. Delayed blastocysts were activated by injecting oestradiol-17β to delayed implanting mice or by exposure to culture medium lacking serum. Four enzymes of carbohydrate metabolism (phosphofructokinase, pyruvate kinase, lactate dehydrogenase, malate dehydrogenase) were studied at different time periods and specific time-dependent changes were found during activation. A characteristic feature was a marked decline by 6 h in the activities of phosphofructokinase and pyruvate kinase. Blastocyst activation in vitro and in utero showed similar trends in metabolic patterns during the 24-h period studied.

Introduction

Mouse blastocysts are known to be metabolically less active during the prolonged free-living phase associated with delayed implantation than they are during normal implantation (McLaren, 1973; Van Blerkom, Chavez & Bell, 1979). Oestrogen given to animals with delay of implantation induces embryonic activation and implantation (Yoshinaga & Adams, 1966). The mechanism by which delayed implanting blastocysts are rendered dormant and then reactivated to implant at a later time is unknown. It has been suggested that factors in uterine fluid such as proteins (Surani, 1975), proteolytic enzymes (Hoversland & Weitlauf, 1978) and glucose (Nilsson, Ostensson, Eide & Hellerstrom, 1980) are stimulatory regulators of blastocyst metabolism. However, metabolic activation can occur in vitro in the absence of any stimulatory factor such as serum (Weitlauf & Kiessling, 1981). Studies on carbohydrate metabolism during delay of implantation suggest that there is a depression of metabolic functions in the quiescent blastocyst, with reduced CO₂ output (Menke & McLaren, 1970) and oxygen consumption (Nilsson, Magnusson, Widehn & Hillensjo, 1982). Nevertheless, there are elevated levels of phosphofructokinase, pyruvate kinase and low lactate dehydrogenase in delayed mouse blastocysts, which returned to normal preimplantation levels upon activation with oestradiol-17β (Sahuja, Sengupta & Manchanda, 1982). It is not clear whether this represents heightened function of glycolysis in the delayed blastocysts or whether the enzymes showed high levels but may not have been in an active state. The present study was undertaken to study the time course of metabolic activation in blastocysts after exposure to culture medium lacking serum and comparing these events to activation occurring in utero after oestrogen injection to mice with delay of implantation.

Materials and Methods

Adult, virgin, Swiss mice weighing 20–25 g were maintained in 14 h light/24 h (lights on 05:00–19:00 h) and fed ad libitum on standard pellet diet and water. The females were caged overnight with males of proven fertility and the day of finding the vaginal plug was designated as Day 1 of
pregnancy. The pregnant females were allotted to 3 groups. In Group 1, animals were left intact (i.e. normal implantation). In Group 2, animals were bilaterally ovariectomized on Day 3 and injected with progesterone (Sigma, St Louis, MO, U.S.A., 2 mg/day) until Day 8 (i.e. ‘delayed implantation’). In Group 3, animals were bilaterally ovariectomized on Day 3, given progesterone (2 mg/day) until Day 8 and then a single injection of oestradiol-17\(\beta\) (Sigma), 30 ng, s.c., on Day 8 (i.e. activation in utero).

Group 1 animals were killed on Day 4 at 22:00 h and the uteri were flushed with Earle’s balanced salt solution containing 0-1% bovine serum albumin (BSA; Sigma). The blastocysts were collected for enzyme assays. Group 2 animals were killed on Day 8 and the blastocysts obtained were used either for enzyme assays of ‘delayed blastocysts’ or for incubation in vitro. Group 3 animals were killed on Day 9 at 6, 12, 18 and 22 h after oestrogen injection and the blastocysts were collected for enzyme assays.

**In-vitro incubation.** The blastocysts were incubated for in-vitro activation as described by Weitlauf & Kiessling (1981). The uteri of Group 2 animals were flushed with tissue culture medium (Eagle’s basic medium; Eagle; 1955) containing 1 mg BSA/ml and placed in a Dubnoff shaking incubator at 37°C. The blastocysts were incubated in the medium in an atmosphere of 5% CO\(_2\) in air, for 6, 12, 18 and 24 h and assayed for the enzymes at the end of each incubation period.

**Enzyme assays.** The blastocysts collected from each group were homogenized in the required numbers in all-glass homogenizers (maximum volume 200 \(\mu\)l), in a volume of 100 \(\mu\)l of the specific buffer for each enzyme, containing 0.05% BSA. The enzymes studied were phosphofructokinase (PFK; EC 2.7.1.11) by a modification of the method of Ling, Paetkau, Marcus & Lardy (1966), pyruvate kinase (PK; EC 2.7.1.40) by a modification of the method of Valentine & Tanaka (1966), lactate dehydrogenase (LDH; EC 1.1.1.27) by the method of Brinster (1965) and malate dehydrogenase (MDH; EC 1.1.1.37) by a modification of the method described in the information bulletin of Boehringer Mannheim, West Germany. The methods were initially modified and then standardized for embryo enzyme assays using a total assay volume of 1 ml. Preliminary experiments were carried out to determine the optimum number of blastocysts required to give a linear change in optical density over a period of 45 min. The activities of PFK and PK could be determined in a minimum of 10 embryos for each replicate. The decrease in A\(_{340}\) for PFK and PK was noted at 28°C and 37°C respectively for a period of 15 min. MDH and LDH activities were determined in a minimum of 2–3 embryos for each replicate. The decrease in A\(_{340}\) was noted for 15 min at 37°C. Controls were run to determine the rate of oxidation of NADH. All chemicals were purchased from Sigma, St Louis, MO, U.S.A. and the enzymes required in the assays were from Boehringer Mannheim (West Germany).

For the various groups at each time interval studied the numbers of cells per blastocyst were determined in more than 30 embryos per group by using the method of Tarkowski (1966).

**Analysis of data.** The statistical significance of differences in enzyme activities was determined between blastocysts undergoing activation in utero and in vitro when expressed per blastocyst and per 100 cells in comparison to ‘normal implanting’ blastocysts by using the Student–Newman–Keuls multiple range test (Newman, 1939).

**Results**

**Cell numbers**

It was seen that in delayed blastocysts the number of cells per blastocyst was significantly higher than in normal implanting blastocysts (109.43 ± 4.41 compared with 91.97 ± 2.71). After activation in utero there was a significant increase in cell numbers (160.15 ± 6.37) while in-vitro activation did not result in any significant increase (121.45 ± 4.66).
**Enzyme activities**

The blastocyst enzyme activities during activation *in utero* and *in vitro* are given in Table 1 and summarized in Text-fig. 1.

**Table 1.** Enzyme activities in mouse blastocysts in Groups 1, 2 (activated *in vitro*) and 3 (activated *in utero*)

<table>
<thead>
<tr>
<th>Group</th>
<th>PFK</th>
<th>PK</th>
<th>LDH</th>
<th>MDH</th>
<th>PFK</th>
<th>PK</th>
<th>LDH</th>
<th>MDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol NADH oxidized/h/blastocyst</td>
<td></td>
<td></td>
<td></td>
<td>nmol NADH oxidized/h/100 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (Day 4)</td>
<td>1.86 ± 0.14 (9)</td>
<td>1.16 ± 0.11 (6)</td>
<td>7.01 ± 0.40 (9)</td>
<td>0.85 ± 0.05 (6)</td>
<td>2.02 ± 0.15</td>
<td>1.26 ± 0.12</td>
<td>7.61 ± 0.43</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>0 h</td>
<td>3.12 ± 0.10* (6)</td>
<td>1.51 ± 0.16* (6)</td>
<td>3.06 ± 0.17* (6)</td>
<td>0.77 ± 0.08 (6)</td>
<td>2.85 ± 0.18*</td>
<td>1.38 ± 0.14</td>
<td>2.80 ± 0.16*</td>
<td>0.70 ± 0.07*</td>
</tr>
<tr>
<td>6 h</td>
<td>1.79 ± 0.15† (6)</td>
<td>1.02 ± 0.06† (6)</td>
<td>3.56 ± 0.47† (6)</td>
<td>0.82 ± 0.08 (7)</td>
<td>1.65 ± 0.14†</td>
<td>0.95 ± 0.05†</td>
<td>3.28 ± 0.43†</td>
<td>0.76 ± 0.06</td>
</tr>
<tr>
<td>12 h</td>
<td>1.52 ± 0.12† (6)</td>
<td>1.00 ± 0.06† (6)</td>
<td>3.95 ± 0.30† (6)</td>
<td>0.77 ± 0.05 (6)</td>
<td>1.29 ± 0.04†</td>
<td>0.85 ± 0.05†</td>
<td>3.34 ± 0.25†</td>
<td>0.64 ± 0.05†</td>
</tr>
<tr>
<td>18 h</td>
<td>1.66 ± 0.06† (6)</td>
<td>1.08 ± 0.14† (6)</td>
<td>4.85 ± 0.35† (7)</td>
<td>0.77 ± 0.06 (6)</td>
<td>1.47 ± 0.05†</td>
<td>0.95 ± 0.12†</td>
<td>4.30 ± 0.31†</td>
<td>0.68 ± 0.05†</td>
</tr>
<tr>
<td>24 h</td>
<td>2.01 ± 0.08† (6)</td>
<td>1.20 ± 0.09 (6)</td>
<td>5.96 ± 0.70† (6)</td>
<td>0.75 ± 0.07 (6)</td>
<td>1.66 ± 0.07†</td>
<td>0.99 ± 0.07†</td>
<td>4.91 ± 0.06†</td>
<td>0.62 ± 0.06†</td>
</tr>
<tr>
<td>3 h</td>
<td>3.12 ± 0.20* (6)</td>
<td>1.51 ± 0.16* (6)</td>
<td>3.06 ± 0.17* (6)</td>
<td>0.77 ± 0.08 (6)</td>
<td>2.85 ± 0.18*</td>
<td>1.37 ± 0.14</td>
<td>2.80 ± 0.16*</td>
<td>0.70 ± 0.07*</td>
</tr>
<tr>
<td>6 h</td>
<td>1.55 ± 0.10† (6)</td>
<td>1.13 ± 0.05† (6)</td>
<td>3.01 ± 0.53† (6)</td>
<td>0.90 ± 0.16 (6)</td>
<td>1.21 ± 0.08†</td>
<td>0.88 ± 0.04†</td>
<td>2.35 ± 0.41†</td>
<td>0.70 ± 0.12*</td>
</tr>
<tr>
<td>12 h</td>
<td>1.53 ± 0.08† (6)</td>
<td>1.07 ± 0.08† (6)</td>
<td>4.08 ± 0.41† (6)</td>
<td>1.02 ± 0.10 (6)</td>
<td>1.16 ± 0.06†</td>
<td>0.81 ± 0.06†</td>
<td>3.09 ± 0.31†</td>
<td>0.77 ± 0.08</td>
</tr>
<tr>
<td>18 h</td>
<td>1.55 ± 0.09† (7)</td>
<td>1.04 ± 0.17† (7)</td>
<td>4.51 ± 0.29† (12)</td>
<td>0.82 ± 0.07 (6)</td>
<td>1.03 ± 0.06†</td>
<td>0.69 ± 0.11†</td>
<td>2.99 ± 0.19†</td>
<td>0.54 ± 0.04†</td>
</tr>
<tr>
<td>22 h</td>
<td>1.94 ± 0.22† (7)</td>
<td>1.19 ± 0.13 (7)</td>
<td>4.99 ± 0.24† (10)</td>
<td>0.79 ± 0.07 (6)</td>
<td>1.21 ± 0.13†</td>
<td>0.74 ± 0.08†</td>
<td>3.11 ± 0.15*</td>
<td>0.49 ± 0.18§</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. for the no. of replicates indicated in parentheses.

*Values significantly different (P < 0.05) from those in Group 1 (*), the 0-h value in Groups 2 or 3 (†), the 6-h value (‡) and the 12-h value (§).*

**Text-fig. 1.** Activity of (a) phosphofructokinase, (b) pyruvate kinase, (c) lactate dehydrogenase and (d) malate dehydrogenase in normal implanting (Group 1), delayed (0 h), activated *in utero* (Group 3) and activated *in vitro* (Group 2) blastocysts. Values are mean ± s.e.m. for the numbers given in Table 1.
Discussion

The present study shows specific time-dependent changes in the activities of phosphofructokinase, pyruvate kinase, lactate dehydrogenase and malate dehydrogenase in dormant blastocysts undergoing activation in utero after oestradiol-17β injection and after exposure to culture medium lacking serum. Our results demonstrate that removal of dormant blastocysts from the maternal uterine milieu (Group 2) initiates a set of metabolic events having a time course similar to that in utero after activation with oestradiol-17β injection (Group 3). As found by Weitlauf & Kiessling (1980), our results show that metabolic activation in vitro is not accompanied by an increase in cell numbers, while activation in utero resulted in a significantly higher number of blastomers. Our earlier study (Sakhuja et al., 1982) had shown that dormant blastocysts exhibited significantly higher levels of PFK and PK, in comparison to normal implanting or delayed and activated blastocysts. We have now observed that within 6 h after activation in utero and in vitro there was a sharp decline in PFK and PK levels and, thereafter, steady states were maintained during the next 18 h, the levels at this time being similar to those found in normally implanting embryos. LDH activity was significantly lower in delayed blastocysts. A marked, but gradual increase in the measurable levels of LDH was seen during in-vitro activation and by 24 h the enzyme activity had increased to reach the values found in normal implanting blastocysts. Even when expressed per 100 cells as well as on a whole blastocyst basis, LDH activity was significantly higher by 18 and 24 h after activation, but only after in-vitro activation was there net synthesis of the enzyme since there was no corresponding increase in cell numbers. After activation in utero and in vitro the levels of MDH showed a decline only on cell number basis.

We are unable to speculate upon the significance of these observations in terms of possible different rates of metabolic activation in delayed blastocysts occurring in utero and in vitro. The rates of metabolic processes depend not only on the amount of enzyme present in the cell but also upon the catalytic efficiency of the enzyme (Dixon & Webb, 1979). The observed changes in metabolic enzymes in the present study could reflect net synthesis or reactivation of enzyme units by substrate cofactors which could in turn control glucose utilization by embryonic cells (Barbehenn & Wales, 1978).

Studies on metabolism during activation of delayed blastocysts have shown changes within 4–6 h in oxygen consumption (Nilsson et al., 1982), protein synthesis (Weitlauf, 1974a, b) and mRNA accumulation (Weitlauf, 1982). We now report that changes in regulatory enzymes of carbohydrate metabolism are also observable within 6 h. Our present findings on metabolic reactivation of blastocysts exposed to culture medium containing BSA as the only macromolecular component lend further support to the earlier reported results on activation of CO₂ production (Torbit & Weitlauf, 1975), amino acid accumulation (Weitlauf, 1973; Van Winkle, 1981) and DNA synthesis (Given & Weitlauf, 1982) in similarly activated delayed blastocysts. It has been suggested that the metabolic dormancy of blastocysts during delayed implantation is due to an inhibitory influence of factors present in the uterine environment (McLaren, 1973; Psychoyos, Bitton-Casimir & Brun, 1975; Weitlauf, 1978). Activation in vitro in the absence of any potential maternal influence or any specific stimulatory factors contained in serum probably occurs as a consequence of removal of the blastocyst from the inhibitory environment of a 'delayed' uterus.

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References


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