Hexokinase Is a Key Enzyme of Glucose Metabolism Expressed during Preimplantation Mouse Development

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Abstract: *Glucose incorporation and utilization in mouse embryos increases during preimplantation development, which may depend at least in part on the hexokinase activity in the embryos. Microdetermination methods including NADP cycling were used to quantitatively examine the enzymatic activity of hexokinase. Hexokinase activity in 1-cell embryos was low (0.035 ± 0.010 pmol of NADPH formed/embryo/min), but progressively increased during preimplantation development. Although there is a significant delay, this increase is also observed when 2-cell embryos are developed in vitro. This increase in hexokinase activity was inhibited by the administration of actinomycin-D in the medium. A reverse transcription-polymerase chain reaction was used to study the expression of hexokinase mRNA in preimplantation mouse embryos. Messenger RNA was obtained from 100 of 2-cell embryos and blastocysts using the Micro-Fast Track mRNA isolation kit. Hexokinase mRNA is detectable after the 2-cell embryo stage. The levels of mRNA increased during embryonic development. These results suggest that hexokinase may be a key enzyme synthesized as the expression of zygotic genome in preimplantation embryos, and help in assessing the quality of embryos developed in vitro.*

Key words: *Glucose, Hexokinase, mRNA, Reverse transcription-polymerase chain reaction, Enzymatic cycling.*

Glucose is unable to support the development of oocytes and early embryos until the 4-cell stage [1]. There is a characteristic switch in substrate preference of the 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. There is a substantial amount of information indicating a change in the uptake or metabolism of glucose in early embryos [3–5]. Low activity of the enzyme hexokinase has been proposed to explain the inability to utilize glucose in the mouse embryo [6–10]. We have also shown that the activity of hexokinase is low in human, mouse, and rat oocytes [11, 12].

In this study, we employed microtechniques and a microassay method to determine the developmental changes in hexokinase activity, and the differences noted during the preimplantation period between *in vivo* and *in vitro* development. The main purpose of this study was to determine whether hexokinase mRNA is developmentally expressed in embryos, and therefore may be responsible for hexokinase expression and glucose utilization by the preimplantation embryo. In order to detect small amounts of mRNA, the reverse transcription-polymerase chain reaction (RT-PCR) method, which is another sensitive technique because of its exponential amplification process, has been employed to study hexokinse expression in preimplantation mouse embryos.

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Materials and Methods

Mouse embryos: Eight-week-old Crj:CD-1 (ICR) female mice were superovulated with 5 IU of pregnant mare serum gonadotropin (Teikokuzoki, Tokyo), followed 48 h later by 5 IU of human chorionic gonadotropin (hCG; Mochida, Tokyo). Mating with males of the same strain was confirmed by the presence of a vaginal plug.

Fertilized ooyctes, 2-cell embryos, 8-cell embryos and blastocysts were obtained at 26, 40, 64 and 88 h after hCG administration, respectively, by flushing the oviducts or the uterii. Randomely selected 2-cell embryos were placed in 2 ml of modified Biggers-Whitten-Wittingham (mBWW) medium [13] in Petri dishes, and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 48 h to obtain embryos developed to blastocysts *in vitro*. Actinomycin-D (Sigma, St. Louise, U.S.A.) dissolved in 1 μl of distilled water was added in the medium at the final concentrations of 1–10 ng/ml. As a control, embryos were cultured in the medium alone.

Hexokinase assay procedures: All embryos from each treatment were washed 3 times with PBS and collected together in 5 μl of PBS and placed on glass slides. They were freeze-dried overnight in a vacuum drying tube at −35 °C at a vapor pressure of 0.01 mm Hg or less.

Hexokinase (EC 2.7.1.1) activities were assayed under the following reaction conditions according to slight modifications of the method reported by Kato and Lowry [14] and Tsutsumi *et al.* [12]. The assay mixture consisted of 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5% Triton X-100, 5 mM glucose, 6 mM ATP, 0.6 mM $NADP^+$ (Boehringer and Sohn, Mannheim, Germany), glucose 6-P dehydrogenase 0.9 μg/ml. A small volume (1 μl) of assay mixture was placed as a droplet at the bottom of a small paraffin oil well (oil well technique; [15]). The enzyme reaction was started by adding the embryo to each droplet of the assay mixture under a microscope. Each enzyme reaction was allowed to proceed for 60 min at 20 °C. As controls, the assay mixtures with embryo in which the substrate was omitted and those without embryo were processed in parallel with the assay mixtures containing embryos. The reaction was stopped by adding 0.2 M NaOH to the droplet. Then the oil well rack was heated at 70 °C for 30 min to destroy excess $NADP^+$ in the hexokinase assays. This treatment does not destroy the NADPH formed in these assays [15]. Subsequently, a 1.5 μl aliquot of the droplet mixture was added to 50 μl of NADP cycling reagent [15, 16] a 10,000-fold amplification [12]. As cycling controls, standard concentrations of NADPH were added to the assay mixture droplets and processed in parallel with the assay mixtures containing embryos. Enzyme activities were expressed as NADPH produced either per embryo or per kg dry weight calculated from the weight of each embryo.

RNA extraction: One hundred embryos were washed five times with phosphate buffered saline containing 3 mg/ml polyvinylpyrroridone. They were collected in a small droplet (<5 μl) and were quick-frozen in liquid nitrogen and stored at −80 °C. $PolyA^+$ RNA was extracted from the embryos by the RNA Zol B mRNA isolation kit (Biotech Lab. Inc. Houstom, Texas, USA), which allowed isolation of $polyA^+$ RNA directly from a very small number of cells. Briefly, the lysates of 100 embryos were directly applied to oligo (dT) cellulose for adsorption. Non-polyadenylated RNA, DNA, dissolved membranes, proteins and cellular debris were washed off the resin with a high salt buffer, and tRNA and rRNA were eluted with a low salt buffer. Finally, the polyadenylated RNA was eluted with water and ethanol precipitated. The resulting pellet of $polyA^+$ RNA, which was obtained by centrifugation, was resuspended in 4 μl of 10mM Tris-HCl, pH 7.6, 1mM EDTA, and 2 μl were used for each of two RT-PCR reactions; one was for the hexokinase mRNA assay, and the other for β-actin mRNA as an internal standard.

Reverse transcription-polymerase chain reaction: Each 2μl of $polyA^+$ RNA aliquot was incubated with 2.5 units of rTth DNA polymerase (Toyobo, Japan) for 15 min at 70 °C in a total reaction volume of 10μl containing 1XrTth reverse transcriptase buffer (10 mM Tris-HCl, pH 8.3, 90 mM KCl), 0.2 mM of each dNTP, 1mM $MnCl_2$ and 10 pmol of the appropriate antisense primer. The reverse transcription reaction was stopped by placing the tube on ice. PCR was performed in the same tube after adding 40 μl of chelating buffer (5% glycerol, 10 mM Tris-HCl, pH 8.3, 100 mM KCl, 0.75 mM EGTA, 0.05% Tween 20) containing 1.8 mM $MgCl_2$ and 10 pmol of the appropriate sense primers. The primers for the amplification of a hexokinase-specific sequence [17] and those for mouse β-actin [18] are shown in Table 1. PCR was performed in a DNA thermal cycler (PC-800 ASTEC LTD.) according to the following protocol: an initial denaturation at 95 °C for 1 min was followed by 40 cycles of denaturation at 94 °C for 1 min, and annealing, and primer extension at 58 °C for 1 min. A further 2 min extension at 72 °C was performed at the end of the 35 cycles to extend any remaining single strand products. Ten microliters of the PCR amplified products were electrophoresed in a 2% agarose gel containing 0.5 μg/ml ethidium bromide and photographed under UV-irradiation.

Statistical analysis: Results are shown as mean ± SD. Statistical analysis was performed using the Student's *t*-test unless otherwise stated.

Results

The hexokinase activity in fertilized oocytes (1-cell embryos) 28 h after hCG administration was 0.035 ± 0.010 pmol of NADPH/embryo/min. The hexokinase activity significantly increased during embryonic development. Developmental changes of hexokinase activity in embryos obtained from superovulated mice are shown in Fig. 1. Overall there was an 8- to 10-fold increase in hexokinase activity during the first 72 h of embryonic development. A significant increase is observed between 40 and 64 and 64 and 88 h after hCG treatment when the embryos developed to 8-cells and blastocysts.

The hexokinase activity of blastocysts developed *in vitro* showed increases after culture paralleling the rise in embryos developed *in vivo*. When development was compared between embryos growing *in vivo* and *in vitro*, hexokinase activity levels of embryos at the same age, 88 h after hCG developed *in vitro* were significantly lower than in embryos developed *in vivo*; only that of *in vitro* developed blastocysts was two-thirds of *in vivo* developed blastocysts. Morphologically, both types of embryos were blastocysts and could not be distinguished by their appearance. Actinomycin-D inhibited the increases of hexokinase activity in embryos developed *in vitro* dose-dependently and significantly (Fig. 2).

Messenger RNA was isolated from batches of embryos at the 2-cell and blastocyst stages of development. The mRNA was primed with oligo-dT and reverse transcribed. An aliquot of the product was used for amplification by PCR for each primer pair specific for actin cDNA or for hexokinase cDNA. The assays were repeated three times with different embryo batches. The identity of the amplified products was confirmed by its size on an agarose gel and its restriction enzyme cleavage pattern. The RT-PCR products from mouse embryos are shown in Figs. 3 and 4. Hexokinase mRNA was not detectable in fertilized oocytes but it was detected in both the 2-cell embryos and blastocysts as evidenced by the presence of amplified cDNA fragments of the expected 361 bp size (Fig. 3). Repeated analysis of the intensity of the amplified fragments indicated that a relatively larger amount of mRNA is produced in the blastocysts than the 2-cell embryos. Amplified β-actin cDNA of the expected 243-bp size was also detected in

Table 1. Primers used in the reverse transcription-polymerase chain reaction assay for hexokinase and β-actin in embryos

Gene	Primers used	Product size
Hexokinase	5′-GAACCACGAGAAGACTCAGAA	375 bp
	3′-CTGAACGCCGATGCTACTGGT	
β-actin	5′-TGTATTCCCCTCCATCGTGG	301 bp
	3′-CAAACTCTGGAAGTTGTGGG	

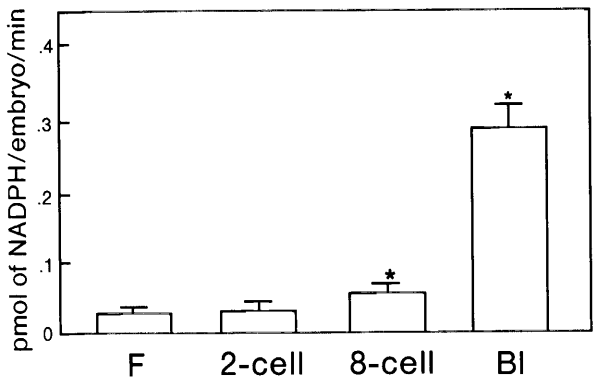

Fig. 1. Developmental changes of hexokinase activity in mouse embryos. All values represent mean ± SD pmol NADPH formed/embryo/min at 20 °C for 8 to 12 embryos. *Significantly different from earlier stage embryos (P<0.01). F: fertilized oocyte, Bl: blastocyst.

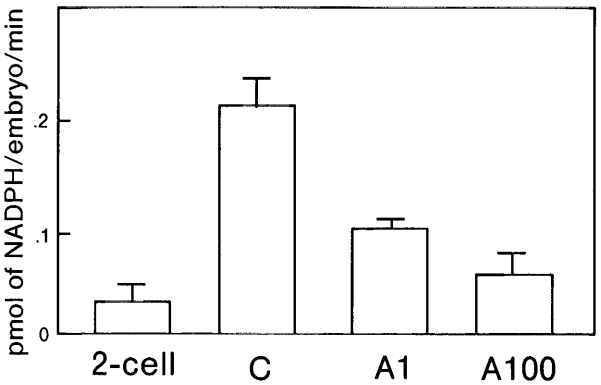

Fig. 2. Comparison of the activities of hexokinase in blastocysts developed *in vitro* with or without actinomycin-D. C: blastocyst developed *in vitro* from 2-cell stage. A1: blastocyst developed with 1 ng/ml of actinomycin D. A100: blastocyst developed with 100 ng/ml of actinomycin D.

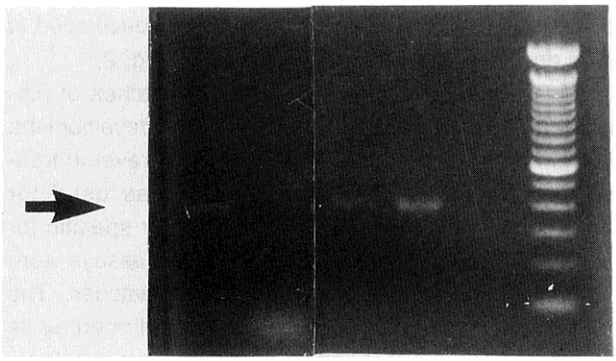

Fig. 3. Detection of hexokinase transcripts in preimplantation embryos by RT-PCR. Lane G, granulosa cells; Lane 1, fertilized oocytes; Lane 2, two-cell embryos; Lane 3, blastocysts; Lane M, molecular weight markers. The position of the expected 361 bp PCR product for hexokinase is indicated.

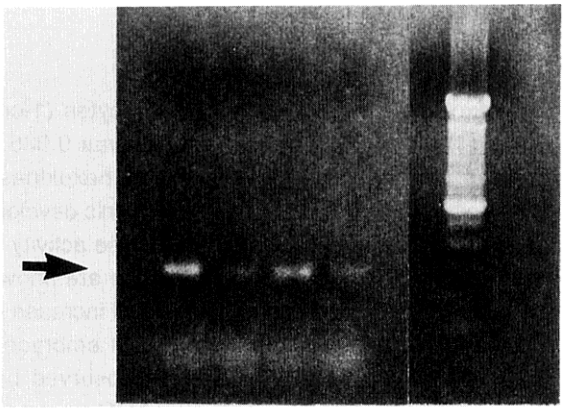

Fig. 4. Detection of β-actin transcripts in preimplantation embryos by RT-PCR. Lane G, granulosa cells; Lane 1, fertilized oocytes; Lane 2, two-cell embryos; Lane 3, blastocysts; Lane M, molecular weight markers. The position of the expected 301 bp PCR product for ß-actin is indicated.

fertilized oocytes, the 2-cell embryos and blastocysts (Fig. 4). No difference was observed in the amount of this PCR product in the embryos.

Discussion

The present microanalysis of single embryos has not only shown changes in enzyme activity occurring during development both *in vivo* and *in vitro* but also a difference of the activity between individual embryos. This may be useful for biochemically analyzing preimplantation embryonic development, since morphologic criteria such as cell division and blastocyst formation are not quantitative and of limited utility. The enzymatic activities of hexokinase were similar to those of superovulated mouse embryos previously assayed in groups of 10 to 25 embryos [1] and in single embryos [10] and showed a significant increase during the preimplantation period as reported here (Fig. 1) and previously [6, 10, 11, 19].

The hexokinase activity in 1-cell mouse embryos was remarkably low when compared with those of somatic organs analysed by the same assay procedure [19]. In earlier study of metabolites the block of glucose utilization at the phosphofructokinase step was proposed because of the low level of fructose 6-P [7, 8]. Because hexokinase catalyzes the phosphorylation of glucose, only a minute amount of glucose 6-P and fructose 6-P would be available for metabolism, suggesting that glucose utilization in embryos is likely to be limited at this step of glycolysis. This may be at least partially responsible for one characteristic feature of early embryos from rodents to primates, the inability to use glucose as an energy source to support their development [1].

The levels of hexokinase activity increased remarkabley during preimplantation development. This change may explain the increased capacity of glucose utilization of the blastocyst, and the switch in substrate preference from pyruvate to glucose. Interestingly, the activity of hexokinase in the blastocysts developed *in vitro* is significantly lower than in blastocysts developed *in vivo*. These blastocysts are not distinguishable by their appearance. This discrepancy may explain the difference in the quality of embryos developed *in vitro* or *in vivo*, suggesting that hexokinase activity measures could be employed to study the preimplantation development of embryos. Actinomycin-D, which is the inhibitor of protein synthesis at the level of transcription, inhibited the increases of hexokinase activity in embryos (Fig. 2). It is conceivable that the increases in hexokinase activity may depend on the protein synthesis as the new expression of embryonic genome during development probably after the 2-cell stage.

Purified mRNA was isolated from mouse embryos at various stages of development by our RNA extraction procedure. Contamination of genomic DNA was ruled out by PCR amplification of each cDNA preparation us-

ing primers that flank a target sequence of the actin gene that contains an 87 bp intron. If genomic DNA were present in the cDNA, a 330 bp target sequence would be amplified as well as the 243 bp fragment representing the cDNA. No 330 bp PCR product for β-actin was detected in any of the RT-PCR reactions with embryo RNA samples (Fig. 4). This RT-PCR assay, however, is not quantitative enough to confirm the reported increase in actin mRNA levels which was detected by Northern blot analysis using thousands of embryos [20].

We have shown that mouse embryos express hexokinase using RT-PCR on mRNA from 50 embryos (Fig. 3). Hexokinase is the likely rate-limiting enzyme of glucose utilization in early mouse embryos. Moreover, the levels of hexokinase activity increase (Fig. 1). This offers some insight into the characteristic switch in the substrate preference of embryos from pyruvate to glucose. The elevated levels of hexokinase expression may be responsible for this increased enzyme activity of hexokinase, which may be related, at least in part, to substrate preference. It is also possible, however, that increased glucose transporter GLUT1 expression may explain the switch in substrate preference from pyruvate to glucose, and may correlate with the exponential increase in glucose incorporation during preimplantation embryo development [21, 22]. In conclusion, the determination of hexokinase activity and its mRNA expression may be useful for investigating not only developmental changes, but also the possible involvement of certain factors influencing embryonic development.

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マウス初期胚においてHexokinaseはグルコース代謝の律速酵素であり活性変化は胚の遺伝子発現による

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マウス初期胚のエネルギー代謝は特異的でグルコースは初期の胚では利用性が不良であるが8細胞期以降の胚はグルコースを利用可能である．その原因としてhexokinase活性の変化が考えられている．サイクリング法でマウス受精卵のhexokinase 活性を測定すると0.035±0.010 pmol of NADPH formed/embryo/minと低活性で，胚発育に伴い増加し胞胚では約10倍に達した．また活性上昇は胚を体外培養することにより観察されたが，蔬態的に同一な胞胚でも *in vivo* 胚に比べ *in vitro* 胚では60%程度の活性値にとどまった．またactinomycin-D添加で活性の上昇は抑制された．RT-PCR法によりhexokinase mRNA を検出したところ，2細胞期胚よりメッセージの発現を認め，発育に伴い増強した．これらの成績より初期胚においてhexokinaseはグルコース代謝の律速酵素でその活性は発現はmRNAレベルで調節され，胚発育に伴い増加すると考えられる．

キーワード：グルコース，Hexokinase，RT-PCR法，酵素サイクリング法．