Molecular and Cellular Endocrinology 333 (2011) 28–36

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Altered glucose transport to utero-embryonic unit in relation to delayed embryonic development in the Indian short-nosed fruit bat, *Cynopterus sphinx*☆

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ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form
26 November 2010
Accepted 27 November 2010

Keywords:
Delayed embryonic development
Glucose
Glucose transporters (GLUTs)
Fat

ABSTRACT

The aim of this study was to compare the changes in concentration of glucose and glucose transporters (GLUTs) in the utero-embryonic unit, consisting of decidua, trophoblast and embryo, during delayed and non-delayed periods to understand the possible cause of delayed embryonic development in *Cynopterus sphinx*. The results showed a significantly decreased concentration of glucose in the utero-embryonic unit due to decline in the expression of insulin receptor (IR) and GLUT 3, 4 and 8 proteins in the utero-embryonic unit during delayed period. The *in vitro* study showed suppressive effect of insulin on expression of GLUTs 4 and 8 in the utero-embryonic unit and a significant positive correlation between the decreased amount of glucose consumed by the utero-embryonic unit and decreased expression of GLUTs 4 ($r = 0.99$; $p < 0.05$) and 8 ($r = 0.98$; $p < 0.05$). The *in vivo* study showed expression of IR and GLUT 4 proteins in adipose tissue during November suggesting increased transport of glucose to adipose tissue for adipogenesis. This study showed increased expression of HSL and OCTN2 and increased availability of L-carnitine to utero-embryonic unit suggesting increased transport of fatty acid to utero-embryonic unit during the period of delayed embryonic development. Hence it appears that due to increased transport of glucose for adipogenesis prior to winter, glucose utilization by utero-embryonic unit declines and this may be responsible for delayed embryonic development in *C. sphinx*. Increased supply of fatty acid to the delayed embryo may be responsible for its survival under low glucose condition but unable to promote embryonic development in *C. sphinx*.

1. Introduction

The duration of pregnancy in most species of chiroptera is much longer than one would predict on the basis of body size. The prolonged gestation is due in part to an arrest in embryonic development that can last from a few days to several months (Heideman, 2000). Post-implantation embryonic diapause is unique among mammals, having been described only in a few species of bats (Mead, 1993; Krishna, 1999). The embryonic diapause in these bats is under maternal control and is mostly influenced by environmental factors, such as a decline in food availability and low ambient temperature (Racey, 1982; Banerjee et al., 2007). The delayed embryonic development in *Miniopterus schreibersii* coincides closely with the period of winter dormancy (Kimura and Uchida, 1983) and proposed to be due to lowered cellular metabolism (Wimsatt, 1969). In *Pipistrellus pipistrellus*, *Myotis myotis* and *Corynorhinus rafinesque*, the period of slow embryonic development coincided with the period of low environmental temperature (Pearson et al., 1952; Racey, 1969).

The present study was undertaken in the fruit bat, *Cynopterus sphinx*, which breeds twice in quick succession at Varanasi, India. Its gestation period varies significantly in the two successive pregnancies of the year. The first (winter) pregnancy is initiated between late October and early November and lasts for about 150 ± 4 days, whereas the second (summer) pregnancy is initiated in April and lasts for about 125 ± 5 days (Krishna and Dominic, 1983). As it is demonstrated in other bats, *C. sphinx* undergo winter dormancy induced by low ambient temperature and food deprivation, which in turn acts to retard the progress of embryonic development at gastrula stage during November–December (Meenakumari and Krishna, 2005). Similar delay in embryonic development during the early gastrula stage has earlier been described in *Carollia perspicillata* (Rasweiler and Badwaik, 1997). An increase in body mass due to fat accumulation was also demonstrated coinciding with the period of embryonic diapauses in *C. sphinx* (Banerjee et al., 2007). How the changes in fat accumulation (adiposity) affect embryonic development in *C. sphinx* is not known. The deposition of white adipose tissue in bat prior to winter serves as an adaptive measure to meet high-energy requirement during cold.

☆ Supported funding from DST, New Delhi, India.
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doi:10.1016/j.mce.2010.11.033

Insulin has been known as the major physiological regulator of adiposity in mammals (Boswell et al., 1994). A rise in circulating insulin concentration after enhanced eating has been reported (Cosgrove and Foxcroft, 1996). Lipogenesis in adipose tissue is also amplified by insulin. Insulin is also known to regulate cell proliferation of developing blastocyst (Beebe and Kaye, 1991). It promotes development of mammalian preimplantation embryos. In addition to the regulation of glucose transport it exerts mitogenic and anti-apoptotic activities. However, its role in delayed embryonic development has not yet been examined.

From the blastocyst stage onward, adequate transfer of glucose from the mother to the embryo is decisive to the survival and normal development of the embryo in uterus (Moley et al., 1998a,b). Glucose uptake and metabolism are essential for cell proliferation and survival and usually is carried out through glucose transporter (GLUT). During early embryogenesis in mouse and rat, the expression of several GLUT isoforms has been described. GLUTs 1 and 9 were found in all pre-implantation stages, GLUTs 2 and 3 were detected at the 8-cell stage (Hogan et al., 1991; Pantaleon et al., 1997a,b), and GLUTs 4 and 8 at the blastocyst stage onwards (Carayannopoulos et al., 2004). The intracellular localization of GLUT 8 is generally similar to that of the insulin-sensitive glucose transporter GLUT 4 in blastocyst (Carayannopoulos et al., 2000).

C. sphinx exhibit annual cycle of food intake and accumulation of white adipose tissue associated with the use of fat as fuel during winter dormacy (Banerjee et al., 2007). It has been earlier reported that storage of triglyceride in fat cells and expansion of adipose tissue need an adequate supply of both fatty acids and glucose to fat cells (Pagano et al., 2007). Studies in the last few years have shown expression of fatty acid oxidation enzymes, such as carnitine palmitoyl transferase 1, in early embryonic tissue (Oey et al., 2006). This suggests that in addition to glucose, fatty acid oxidation may be important to meet the energy requirement of developing embryos (Berger and Wood, 2004; Oey et al., 2005). Free fatty acid may be obtained from lipolysis of stored fat by the enzyme, hormone sensitive lipase (HSL) (Zechner et al., 2005). L-Carnitine is required for the transport of free fatty acid to mitochondria where beta-oxidation occurs for energy production (Carter et al., 1995). Since the embryo is not capable of substantial carnitine synthesis, maternal circulating carnitine is utilized for materno-fetal transfer of fatty acid (Lahjouji et al., 2004). Recent study suggests that OCTN2 is required for L-carnitine transport (Lahjouji et al., 2004). These findings suggest that the embryo utilizes both glucose and fatty acid as substrate for energy production. However, the changes in the pattern of glucose and/or fatty acid transport to utero-embryonic unit during delayed embryonic development have not yet been examined. The utero-embryonic unit here referred as decidualized (swollen) portion of uterine horn containing embryo from pre-implantation stage onwards.

The aim of the present study was to determine the pattern of glucose and/or fatty acid transport to utero-embryonic unit during the two pregnancies of the year in *C. sphinx* to define their role for delayed embryonic development.

2. Materials and methods

2.1. Source of animals

All experiments were conducted in accordance with the principles and procedures approved by Banaras Hindu University, Departmental Research Committee. The bats (*C. sphinx*) utilized in this study were captured alive between 1998 and 2004 from in and around Banaras Hindu University premises and Ramnagar, Varanasi, India. They were then transported to the laboratory immediately. Body weights of bats were recorded as soon as they brought to the laboratory (within 2 h of capture). Females weighing 43 g or more and having wing-span exceeding 46 cm were sexually mature (Krishna and Dominic, 1983). After the capture from wild the bats were brought to the laboratory and then following a period of 2 h of acclimatization they were sacrificed in between 16.00 and 19.00 h and blood serum was saved and stored at −20 °C until assayed. Location and amount of white adipose tissue deposited in the body of female *C. sphinx* was recorded.

2.2. Chemicals

The antibodies against GLUTs 1, and 3 were kind gift from Prof. Kelle H Moley, Division of Reproductive Endocrinology, Washington University School of Medicine, USA; antibodies against GLUTs 4 and 8 were gifted by Prof. David James, Garvan Institute of Medical Research, Australia and Prof. Jose Emillo Mesonero, Unidad de Fisiologia, Zaragoza respectively; antibody against OCTN2 was gifted by Prof. HK Kroemer, Department of Pharmacology, Germany; antibody against HSL was gifted by Prof. Fredrick Kraemer, Stanford University Medical centre, USA for providing antibody of HSL. Antibody for insulin receptor β-subunit was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Carnitine Acyl carnitine transferase was purchased from Sigma–Aldrich (#C4899, St. Louis, MO, USA). All other general chemicals were purchased from Merck, New Delhi, India.

2.3. Glucose estimation

Glucose was measured from serum and utero-embryonic unit of *C. sphinx* ($n = 45$) using quantitative colorimetric method using the kit obtained from Span Diagnostics Ltd., Mumbai, India. Tissue glucose content from utero-embryonic unit was determined in accordance with Moares et al. (2004). Tissue was homogenized (w/v) in 10 volumes of 6 N perchloric acid (PCA) under ice bath by two 30 s strokes. The acid homogenate was centrifuged at 10,000 × *g* and the supernatant was used for glucose estimation. To 0.02 ml of serum 1.5 ml working glucose reagent (a mixture of phosphate buffer, glucose oxidase peroxidase 4-AP stabilizers-as per manufacturer manual) was added, mixed well and incubated at 37 °C for 10 min. After incubation tubes were again thoroughly mixed and the color intensity was read at 505 nm against purified water. All the tubes were run in duplicates. Intra assay variation was less than 3% and inter assay variation was about 5%.

2.4. Insulin radioimmuno assay (RIA)

Circulating insulin concentration in the female bats ($n = 45$) was measured using the RIA kit supplied by Medicorp Inc., Quebec, Canada (Catalog no: KTSP-11001). Verification of the insulin assay for the use in bat was described previously (Doval and Krishna, 1998). The protocol was used as provided by manufacturer. Briefly, 100 μl of serum sample was added to each insulin antibody-coated tube. To each tube, 1 ml of ^{125}I-insulin was added and incubated for 18 h at room temperature. Finally decanted liquid from the assay tubes except total count tubes was counted for 1 min in Beckman Gamma Counter. Standards, zero and blank tubes were also run along with the samples. The coefficient of intra assay variation was less than 9%.

2.5. In vitro study

Effect of insulin on the utero-embryonic unit: the utero-embryonic unit, the decidualized (swollen) portion of uterine horne containing the embryo in gastrula stage ($n = 9$) was dissected out from the bat during delayed embryonic development in November–December. Both end of utero-embryonic unit was open, so that culture media could easily pass through the uterine lumen containing embryo. The utero-embryonic unit was immediately kept in 1 ml of medium, a mixture of DMEM (with sodium pyruvate and L-glutamine) and Ham's F-12 (1:1; v:v) (Himedia, Mumbai, India) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1% BSA (Sigma), and cultured with low and high dose of insulin (200 and 400 pmol/l). The utero-embryonic unit cultured without insulin was used as control. The plastic culture dishes having the utero-embryonic unit in medium were maintained in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 6 h at 37 °C. Control and treatment group was run in triplicate. Utero-embryonic unit cultured under these conditions appear healthy and do not show any sign of necrosis. Tissues were collected after 6 h, washed several times with PBS and kept frozen at −20 °C for immunoblot study.

2.6. Immunoblot

The utero-embryonic unit (six pooled) was homogenized to produce 10% (w/v) homogenate. Further, protein extraction and immunoblot was performed as described previously (Banerjee et al., 2009). Equal amount of proteins (50 μg) as determined by Folin's method was loaded on to SDS-PAGE (10%) for electrophoresis. Thereafter, proteins were transferred electrophoretically to nitrocellulose membranes (Sigma–Aldrich, St. Louis, MO, USA) overnight at 4 °C. Nitrocellulose membranes were blocked for 60 min with Tris-buffered saline (TBS; Tris 50 mM (pH 7.5), NaCl 150 mM) containing 5% fat-free dry milk and incubated with concerned primary antibodies (GLUTs 1 and 3 at a dilution of 1:500; GLUT 4 at 1:300; GLUT 8 at 1:350; IR at 1:500; HSL at 1:500). Membranes were then washed with three changes of TBS over 10 min. Immunodetection was performed with anti-rabbit IgG–HRP conjugate (1:2000). Finally, blot was washed three times with TBS and developed with enhanced chemiluminescence (ECL) detection system (BioRad, USA). Similarly blot was developed for β actin (Santa Cruz) at dilution 1:1000 as loading control. The densitometric analysis of the blots was performed by scanning and quantifying the