Glucose utilization and the PI3-K pathway: mechanisms for cell survival in preimplantation embryos

Joan K Riley1 and Kelle H Moley1,2

1Department of Obstetrics and Gynecology and the 2Department of Cell Biology and Physiology, Washington University School of Medicine, 4911 Barnes-Jewish Hospital Plaza, St Louis, Missouri 63110, USA

Correspondence should be addressed to KH Moley; Email: moleyk@wustl.edu

Abstract

The maintenance of optimal glucose utilization during the preimplantation period is critical for embryo survival. A decrease in glucose transport during preimplantation development has been linked to the early steps of programmed cell death in these embryos. Decreased glucose transport is not thought to be simply a consequence of cell death, rather it is thought to be a trigger that can initiate the apoptotic cascade. Extensive apoptosis during the preimplantation period may manifest later in pregnancy as a malformation – or miscarriage, if cell loss is excessive. Phosphatidylinositol 3-kinase (PI3-K) is a known regulator of a number of physiologic responses including cellular proliferation, growth, and survival as well as glucose metabolism. Studies performed in other cell systems have demonstrated that the PI3-K pathway plays a critical role in maintaining glucose transport and metabolism. This review will present the current evidence that suggests that PI3-K is vital for preimplantation embryo survival and development. In addition, data demonstrating that PI3-K activity is important for glucose metabolism during this early developmental period will be discussed.

Introduction

Glucose uptake and utilization is vital for embryo survival and development during the preimplantation period. A decrease in glucose uptake during this stage can compromise the developing embryo. The preimplantation period extends from the time of fertilization through the 1-cell, 2-cell and 4-cell stages to the morula and finally to the blastocyst stage. Prior to the blastocyst stage of preimplantation development, murine embryos are unable to metabolize glucose via glycolysis. During the early blastocyst stage, the embryo differentiates from totipotent cells into two cell lineages: the trophoderm (TE) that develops into the placenta and the inner cell mass (ICM) which gives rise to the embryo proper. It is during this time that embryonic metabolism switches from the oxidation of lactate and pyruvate via the Krebs cycle and oxidative phosphorylation to the anaerobic metabolism of glucose through glycolysis (Leese & Barton 1984, Wales 1986). This change in substrates is thought to be due to the biosynthetic and developmental demands placed on the embryo as the blastocyst creates the fluid-filled blastocoel and prepares for implantation.

In eucaryotes, glucose enters a cell by one of two mechanisms. Glucose transport may be an active process in which glucose uptake occurs via sodium coupled glucose transporters (SGLT). The presence of SGLTs during preimplantation embryo development is equivocal and this review will focus on the family of facilitative glucose transporters known as GLUTs. Glucose transport across cell membranes via GLUT proteins is an energy independent process in which glucose is transported down its concentration gradient. Currently, there are thirteen members of the facilitative glucose transporter family, GLUT1–12 and the H+ coupled myo-inositol-transporter (HMIT) (Joost et al. 2002, Wood & Trayhurn 2003). The GLUT family of proteins has been subdivided into three classes: class I consists of GLUT1–4; class II contains GLUT5,7,9,11; and class III consists of GLUT6,8,10,12 and HMIT (see Joost & Thorens 2001, Joost et al. 2002). GLUTs exhibit a high degree of sequence homology, however they differ in their substrate specificity, kinetic characteristics, tissue and subcellular distribution as well as their response to extracellular stimuli. Members of the GLUT family contain an intracellular amino- and carboxy-terminus, 12 membrane spanning domains, a glycosylated extracellular loop and an intracellular loop (Mueckler et al. 1985, Cope et al. 1994).

Facilitative glucose transporters in preimplantation development

Glucose transport in murine preimplantation embryos has previously been attributed to the known facilitative
glucose transporters, GLUT1, GLUT2, and GLUT3 (Hogan et al. 1991, Aghayan et al. 1992, Pantaleon et al. 1997, Moley et al. 1998b) however more recently the expression of a growing number of GLUTs has been detected in preimplantation embryos. The function of these newly identified transporters during the preimplantation period is largely unknown. One of the first transporters to be characterized in preimplantation embryos was GLUT1. GLUT1 mRNA is expressed throughout preimplantation development, namely from the 1-cell through the blastocyst stage in the mouse (Hogan et al. 1991, Aghayan et al. 1992, Morita et al. 1992), rabbit (Robinson et al. 1990), cow (Lequarré et al. 1997, Wrenzyci et al. 1998, Navarrete Santos et al. 2000, Augustin et al. 2001) and human (Dan-Goor et al. 1997). Interestingly, GLUT1 was shown by immunofluorescent confocal microscopy to be expressed in the pronuclei of oocytes and in the nucleus of cleavage stage embryos (Pantaleon et al. 2001). This study suggests an additional yet undefined role for GLUT1 which the authors propose may be related to the physiological state of the cell. While GLUT1 is expressed throughout preimplantation development it is predominantly cytoplasmic until compaction (Pantaleon et al. 2001). In rabbit blastocysts GLUT1 is expressed predominantly at the basolateral surface of the polarized TE cells (Robinson et al. 1990). In contrast, the cellular distribution of GLUT1 was found to be different in murine blastocysts where it was expressed on the apical and basolateral surfaces of the TE cells as well as in intercellular membranes (Aghayan et al. 1992). In addition, it was expressed on the membranes of the ICM. A separate study revealed a more restricted expression pattern for GLUT1 in murine blastocysts namely on the basolateral surface of TE cells and on the plasma membrane of the ICM (Pantaleon et al. 1997). It is hypothesized that the function of GLUT1 in the developing embryo given its cell surface expression in the ICM is to transport glucose into these cells from the embryonic extracellular space (Pantaleon et al. 1997).

GLUT2 expression in preimplantation embryos is controversial. GLUT2 transcripts were detected at the 8-cell/compacted morula stage (Hogan et al. 1991, Schultz et al. 1992) and the protein was detected at the blastocyst stage in mouse embryos (Aghayan et al. 1992, Schultz et al. 1992). In addition bovine embryos were shown to express GLUT2 transcripts during blastocyst elongation at d14 and d16 (Augustin et al. 2001). GLUT2 is reportedly expressed on the basal membrane of TE cells, intracellular vesicles, and on the plasma membrane of the ICM (Aghayan et al. 1992). The function of GLUT2 at these locations in the blastocysts is not yet defined. However it has been speculated that GLUT2 may be responsible for glucose transport into the blastocoel cavity (Pantaleon et al. 1997). Other studies also conducted in mice did not demonstrate GLUT2 expression (Morita et al. 1992, Tonack et al. 2004). In addition, studies conducted in other species namely rabbit and cow did not find GLUT2 to be expressed during the preimplantation period (Augustin et al. 2001, Navarrete Santos et al. 2004a).

GLUT3 transcripts are detected from the 4-cell through the blastocysts stage of murine development (Pantaleon et al. 1997). GLUT3 mRNA has also been detected from the 2/4-cell stage through the blastocyst stage in bovine embryos (Augustin et al. 2001). In mice, GLUT3 protein is detected starting at the late 4-cell stage, where the immunoreactivity is weak and the protein is present in cytoplasmic vesicles (Pantaleon et al. 1997). It remains in vesicles through the 6- and 8-cell stages. In the un compacted morula this protein is present at the plasma membrane. As the embryo develops from the compacted morula to the blastocyst stage, GLUT3 expression is detected on the apical surface of the polarized TE cells. It is thought that the function of GLUT3 in blastocysts is to facilitate the uptake of maternal glucose. In addition, down-regulation of GLUT3 using an antisense oligonucleotide in pooled blastocysts demonstrated a lower percentage of embryos progressing to a blastocyst stage, suggesting that this protein may facilitate blastocyst formation by its ability to transport glucose. Since this treatment did not fully knock down GLUT3 protein expression, it is difficult to conclude that GLUT3 is essential for preimplantation embryo development.

The facilitative fructose transporter, GLUT5, is expressed in but not limited to tissues which are insulin sensitive in both humans and rodents (Shepherd et al. 1992, Kristiansen et al. 1997, Darakhshan et al. 1998, Hajduch et al. 1998) where it transports dietary fructose into cells. Fructose is present in human uterine fluid and thus at the blastocysts stage of development, the early embryo is exposed to this hexose sugar (Casslen & Nilsson 1984). GLUT5 transcripts were not detected in rabbit blastocysts (Navarrete Santos et al. 2004a). However, GLUT5 transcripts can be detected at the 8/16-cell stage in bovine embryos the point at which embryonic genome activation occurs (Augustin et al. 2001). The authors suggest that fructose uptake through GLUT5 in preimplantation embryos may correspond with the shift from the pentose-phosphate pathway towards the production of ribose-5-phosphate which is necessary for nucleotide synthesis.

Most recently, two additional facilitative glucose transporters have been identified in the preimplantation embryo, GLUT9 and GLUT12. Three different isoforms of GLUT9 have been identified in the mouse embryo (Carayannopoulos et al. 2004). The full-length isoform (GLUT9a) contains 12 transmembrane-spanning domains. The two additional isoforms, GLUT9aΔ209–316 and GLUT9bΔ126Δ209–316, are short forms of GLUT9 that contain 10 transmembrane-spanning domains. These isoforms have deleted transmembrane domains 6 and 7 and appear to be splice variants of the same gene. GLUT9aΔ126Δ209–316 contains an alternate aminoterminus but the remainder of the protein is identical to GLUT9aΔ209–316. The short isoforms of GLUT9 have not yet been identified in humans. Two of the GLUT9
isoforms, GLUT9a and GLUT9a\_209–316\_o, have been shown to transport glucose. However, of these two only GLUT9a\_209–316\_o is present in murine blastocysts. GLUT9a\_209–316\_o is expressed at the plasma membrane in 1-cell and 2-cell zygotes and in an intracellular compartment in TE cells at a blastocyst stage. The down-regulation of GLUT9a\_209–316\_o expression using antisense oligonucleotides did not result in decreased glucose uptake in blastocysts nor in the induction of apoptosis. However, when antisense treated embryos were transferred into pseudo-pregnant female mice an increase in pregnancy loss occurred. Thus GLUT9a\_209–316\_o expression is important during early preimplantation development.

GLUT12 is potentially another insulin-sensitive glucose transporter (Rogers et al. 2002). The presence of GLUT12 transcripts was examined at the 2-cell, morula and blastocyst stages of development in murine embryos. GLUT12 expression was strongest at the 2-cell stage and declined thereafter such that the presence of GLUT12 transcripts at the morula and blastocyst stage was very low. Preliminary experiments using an antisense raised against human GLUT12 (Rogers et al. 2002) showed immunoreactivity in 2-cell embryos but not in blastocysts. The promoter region of murine GLUT12 contains sequences that are homologous to known insulin response elements (Zhou et al. 2004). However, whether GLUT12 is an insulin responsive glucose transporter expressed at a protein level in murine embryos remains to be determined.

In murine blastocysts, insulin and IGF-I stimulate glucose uptake through the IGF-I receptor (Gardner & Leese 1988, Harvey & Kaye 1991, Pantaleon & Kaye 1996, Carayannopoulos et al. 2000). Two insulin responsive GLUTs have been identified in preimplantation embryos, namely GLUT8 and GLUT4. In a non-insulin-stimulated state GLUT8 is predominantly located in the cytoplasm of both the ICM and TE in murine blastocysts (Carayannopoulos et al. 2000). Upon insulin stimulation the protein translocates to the plasma membrane of the TE cells. The inhibition of GLUT8 expression via antisense oligonucleotides results in the abrogation of insulin-stimulated glucose uptake in blastocysts. Thus GLUT8 plays a role in insulin-stimulated glucose uptake in murine blastocysts. GLUT8 expression has also been documented in both rabbit and bovine blastocysts. GLUT8 was only expressed at the blastocyst stage in rabbit blastocysts (Navarrete Santos et al. 2004a) whereas it was expressed from the 2-cell through the blastocyst stage in bovine embryos (Augustin et al. 2001). The presence of GLUT4 in mammalian preimplantation embryos is controversival. GLUT4 was not detected in either human or murine preimplantation embryos (Hogan et al. 1991, Aghayan et al. 1992, Schultz et al. 1992, Dan-Goor et al. 1997). In contrast, GLUT4 expression has been reported in other species namely bovine, rabbit, rat and C57BL6 murine blastocysts (Navarrete Santos et al. 2000, 2004a, Augustin et al. 2001, Korgun et al. 2001, Tonack et al. 2004). In murine embryos, GLUT4 is expressed in the cytoplasm of both the ICM and TE where it maintains a perinuclear staining pattern (Tonack et al. 2004). Similar to murine blastocysts, rabbit blastocysts displayed cytoplasmic and perinuclear GLUT4 staining. In addition, GLUT4 expression was detected at the plasma membrane of TE cells and in association with nuclear membranes in the rabbit embryos (Navarrete Santos et al. 2004a). To date however, GLUT4 has not been shown to translocate to the plasma membrane in response to either insulin or IGF-1. Thus it remains to be seen whether GLUT4 is involved in insulin-stimulated glucose uptake in preimplantation embryos.

**Presence and function of PI3-K/Akt in mammalian preimplantation embryos**

Mammalian preimplantation embryos express a number of growth factors and growth factor receptors that are critical during embryo development (for review see Hardy & Spanos 2002). Growth factors have been shown to affect preimplantation embryo gene expression, metabolism and cell death and thus they may have profound effects on embryo development. Insulin and IGF-I are important regulators of cell growth and differentiation and have been shown to have both mitogenic and anti-apoptotic effects on mammalian preimplantation embryos. Studies have demonstrated that the addition of physiologic levels of insulin or IGF-I during in vitro culture results in decreased apoptosis (Herrler et al. 1998, Sapos et al. 2000, Byrne et al. 2002, Makarevich & Markkula 2002, Augustin et al. 2003, Sirisathien & Brackett 2003, Fabian et al. 2004) and or increased cellular proliferation in human, mouse, rabbit and bovine blastocysts (Matsui et al. 1995, Herrler et al. 1998, Byrne et al. 2002, Makarevich & Markkula 2002, Augustin et al. 2003). Insulin treatment of cultured murine and bovine embryos results in increased cell number specifically in the ICM of the developing blastocyst (Harvey & Kaye 1990, Gardner & Kaye 1991, Smith et al. 1993, Sirisathien et al. 2003). Thus insulin and insulin-like growth factors support embryo growth and survival.

Given the anti-apoptotic nature of insulin and IGF-I, inhibition of the IGF-I receptor should lead to increased apoptosis during development. Indeed studies have demonstrated that down-regulation of the IGF-I receptor via exposure to high IGF-I and insulin concentrations results in the induction of apoptosis in murine blastocysts (Chi et al. 2000b). The transfer of the high IGF-I treated blastocysts into pseudo-pregnant female recipient mice revealed increased pregnancy loss (Pinto et al. 2002b). Thus the activation of growth factor receptors, such as insulin and IGF-I, during the preimplantation period in mammals is vital for embryo survival and development. The signal transduction mechanisms by which growth factors mediate their effects in preimplantation embryos are beginning to be elucidated.

A number of growth factor receptors activate phosphatidylinositol 3-kinase (PI3-K). Growth factor activation of
the PI3-K pathway has been reviewed elsewhere (Brazil & Hemmings 2001, Cantley 2002, Thompson & Thompson 2004, Woodgett 2005). PI3-Ks are a family of enzymes that phosphorylate phosphoinositides (Chan et al. 1999). These kinases are divided into three classes, growth factors such as insulin and IGF-I activate class I PI3-Ks. Once activated by cell surface receptors class I PI3-Ks phosphorylate plasma membrane phosphoinositides thus generating docking sites for pleckstrin homology domain containing proteins such as the serine-threonine kinase Akt. It is generally believed that Akt is the primary mediator of the anti-apoptotic signal generated via the PI3-K pathway (Dudek et al. 1997, Khaaja et al. 1997, Philpott et al. 1997, Songyang et al. 1997). PI3-K activity is known to regulate a number of physiologic responses including cellular proliferation, growth, and survival as well as glucose metabolism. Recently, studies have described the expression of PI3-K and its downstream target the serine-threonine kinase Akt in preimplantation embryos.

PI3-K is a heterodimeric enzyme that consists of a p85 regulatory subunit and a p110 catalytic subunit. Murine 2-cell embryos were shown to express the mRNA of multiple PI3-K isoforms including p85a and β and p110 α, β, γ, δ (Lu et al. 2004). In addition, Kawamura et al. (2005) demonstrated the presence of p110 transcripts from the oocyte through the hatched blastocysts stage of murine preimplantation development. PI3-K activity results in the recruitment of Akt to the plasma membrane. Navarrete Santos et al. (2004b) demonstrated the presence of Akt protein in rabbit blastocysts. The Akt protein present in the rabbit blastocysts was phosphorylated indicating that at this stage of development, Akt is likely to be activated by growth factors present in its milieu. A final study examined the expression of both PI3-K and Akt protein throughout murine preimplantation development (Fig. 1). Using pan p85, p110 and Akt antibodies it was determined that both PI3-K subunits and Akt are expressed from the 1-cell through the blastocyst stage of murine preimplantation development (Riley et al. 2005b). These proteins are localized predominantly at the cell surface from the 1-cell through the morula stage. At the blastocyst stage, both PI3-K and Akt exhibited an apical staining pattern in the TE cells. Similar to what Navarrete Santos et al. (2004b) reported in rabbit, Akt was phosphorylated throughout murine preimplantation embryo development and its presence at the plasma membrane is a reflection of its activation status. Most recently, the presence of the PI3-K pathway has also been detected in human blastocysts using a cDNA microarray approach (Adjaye et al. 2005). These studies establish the presence of the PI3-K/Akt pathway in preimplantation embryos and the phosphorylation status of Akt suggests the pathway is active during this developmental period.

Targeted gene deletion experiments initially revealed the importance of the PI3-K pathway during embryo development. The physiologic roles of different classes and isoforms of PI3-K are complex and are beginning to become more well defined. Deletion of certain PI3-K subunits leads to embryonic lethality. Mice deficient in the p110α catalytic subunit of PI3-K die in utero between E9.5 and E10.5 (Bi et al. 1999). The null mice have a proliferation defect (Bi et al. 1999) and multiple vascular defects (Lehierve et al. 2005). Deletion of the p110β subunit leads to early embryonic lethality suggesting a more critical role for this protein during development (Bi et al. 2002). A few p110β deficient embryos were detected at E3.5 (blastocyst stage) however there is a deficit in the number of homozygous knockout embryos detected during this period of development according to Mendelian ratios. Thus both p110α and p110β are vital during embryonic development. The deletion of some PI3-K isoforms does not result in embryonic lethality implying either that these isoforms are not important during development or that functional redundancy exists among these proteins.

The physiologic importance of the PI3-K pathway has recently been documented during the preimplantation period. Lu et al. (2004) demonstrated the importance of the PI3-K pathway in mammalian preimplantation embryo development by showing that the activation of PI3-K by an embryonic trophic factor, platelet-activating factor (PAF), is critical for embryo development and survival. PAF treatment of 2-cell embryos results in a transient increase in calcium that is inhibited by both LY-294002 and wortmannin implying that PI3-K activity is required for this PAF-induced biological response. In addition, a separate global gene expression study demonstrated that genes involved in inositol phosphate and calcium signaling such as Pik3c2a (a class II PI3-K α polypeptide) are increased in activated as compared with dormant blastocysts (Hamatani et al. 2004). The importance of the PI3-K pathway during the preimplantation period was highlighted by a study that demonstrated that inhibition of PI3-K results in decreased numbers of embryos that develop to the morula and blastocyst stage in vitro when cultured from a zygote stage, similar to Pafr deficient embryos (Lu et al. 2004). The blastocysts that did develop contained fewer cells and a larger number of fragmented nuclei. The effects induced by the PI3-K inhibitors were dose-dependent. Interestingly, if the embryos were only exposed to the PI3-K inhibitors from the zygote through the 2-cell stage and then cultured to the blastocyst stage there was again a decrease in cell number and an increase in the number of fragmented nuclei. However, the effect of this limited treatment was not as great as when the embryos were cultured with the inhibitors throughout preimplantation development. Thus activation of the PI3-K pathway, in this case via PAF, is critical for the survival and development of the preimplantation embryo.

Insulin has been shown in other cell systems to activate the PI3-K pathway and thus stimulate glucose uptake by causing the translocation of insulin responsive GLUTs, such as GLUT4, to the plasma membrane (for review see Welsh et al. 2005). It is controversial whether insulin-stimulation results in the activation of this pathway

in preimplantation embryos. Navarrete Santos et al. (2004b) found that insulin-stimulation results in the activation of the mitogen-activated protein kinase (MAPK) pathway but not the PI3-K/Akt pathway in rabbit blastocysts. In contrast, Riley et al. (2005b) demonstrated insulin treatment of murine blastocysts resulted in a 2-fold increase in Akt phosphorylation in comparison with controls. Moreover, LY-294002 and wortmannin were shown to completely inhibit insulin-stimulated glucose uptake at the blastocyst stage (Fig. 2). Thus whether insulin triggers the activation of the PI3-K/Akt pathway and thereby regulates glucose homeostasis in mammalian blastocysts remains to be determined.

Other experiments highlighting the functional importance of the PI3-K pathway during the preimplantation period include those demonstrating that inhibition of the PI3-K pathway results in decreased blastocyst hatching (Riley et al. 2005b). Blastocyst hatching from the zona pellucida is required for successful implantation. Although hatching is delayed in these embryos it does occur as blastocysts that are cultured in vitro for 24 h in the presence of LY-294002 and then transferred back into pseudo-pregnant female recipient mice, resulted in approximately the same

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**Figure 1** Detection of PI3-K subunits, p85 and p110, in preimplantation embryos. Reproduced from Riley et al. 2005b. Preimplantation mouse embryos were retrieved at different stages of development and stained with either preimmune antisera as a negative control or with antibodies specific for the p85 or p110 subunit of PI3-K. The embryos were then incubated with a secondary antibody, Alexa Fluor 488 goat anti-rabbit or donkey anti-goat IgG (green fluorescence). Embryos were counterstained with TO-PRO-3 iodide, which stains nuclei (blue channel).
number of implantation sites but a dramatic increase in the fetal resorption rate as compared with controls (Fig. 3B) (Riley et al. 2005a).

Inhibition of the PI3-K pathway is known to result in the induction of apoptosis in many cell systems. A recent report has shown that inhibition of PI3-K induces apoptosis in murine blastocysts (Gross et al. 2005). Gross et al. (2005) treated in vivo-derived blastocysts with LY-294002 for 24 h and showed a five-fold increase in TUNEL-positive nuclei as compared with controls. Apoptotic cells were detected in both the TE as well as the ICM. Blastocysts cultured for 48 h completely degenerated and displayed rampant apoptosis. We have also cultured in vivo-derived blastocysts for 30 h in vitro, in the presence of increasing concentrations of LY-294002 and demonstrated a dose-dependent increase in the number of TUNEL-positive nuclei per embryo as compared with controls (Fig. 3A) (Riley et al. 2005a). A final study conducted by Kawamura et al. (2005) demonstrated that TGF-α has anti-apoptotic effects on murine preimplantation embryos exposed to suboptimal culture conditions. The mechanism of the anti-apoptotic effect involves the up-regulation of survivin, a member of the inhibitor of apoptosis family. Inhibition of PI3-K using either LY-294002 or wortmannin resulted in the abrogation of TGF-α-induced up-regulation of survivin expression in blastocysts. PI3-K activity is therefore required for the anti-apoptotic effects of TGF-α, as mediated by survivin, in murine preimplantation embryos. Taken together the aforementioned studies demonstrate that PI3-K activity is critical for embryo development and survival during the preimplantation period and that inhibition of this pathway even for discrete periods during preimplantation development has long lasting detrimental effects on embryo development, survival and pregnancy outcome.

Figure 2 Insulin-stimulated glucose uptake is inhibited by LY-294002 and wortmannin. Reproduced from Riley et al. 2005b. Insulin-stimulated glucose uptake was measured in blastocysts exposed to media alone (no treatment), 250 μM LY-294002 and 100 nM wortmannin. The percentage of insulin-stimulated glucose uptake over basal glucose uptake is plotted.

Figure 3 Inhibition of the PI3-K pathway at the blastocysts stage results in increased apoptosis and an increased frequency of fetal resorptions. Reproduced from Riley et al. 2005a. (A) Blastocysts were recovered and cultured in vitro in the presence of DMSO (vehicle control shown is for the highest concentration of LY-294002 used) or increasing concentrations of the PI3-K inhibitor LY-294002. The TUNEL assay was performed and the apoptotic nuclei are depicted in red. Embryos were counterstained with the nuclear dye TO-PRO-3 iodide as shown in blue. (B) Blastocysts were recovered and then cultured in vitro for 24 h in media containing either DMSO or 250 μM LY-294002. Unhatched blastocysts were then transferred back into the uterine horn of pseudopregnant female recipient mice at 2.5 dpc. This panel shows representative uterine horns derived from mice into which blastocysts cultured in either DMSO or LY-294002 were transferred.
Glucose metabolism and apoptosis

Significant progress has been made in recent years linking glucose transport, cell metabolism, the PI3-K pathway, and apoptosis both in preimplantation embryos as well as in other cell systems (for reviews see Moley & Mueckler 2000, Moley 2001, Plas et al. 2002, Plas & Thompson 2002). Apoptosis or programmed cell death in preimplantation embryos is a routine process by which the embryo eliminates abnormal or extraneous cells (for reviews see Hardy 1997, 1999, Jurisicova & Acton 2004, Fabian et al. 2005). Studies demonstrated that in vivo-derived murine and porcine preimplantation embryos commonly do not show TUNEL-positive staining before the blastocyst stage (Long et al. 1998, Kamjoo et al. 2002). Jurisicova et al. (1996) demonstrated in vitro that of 200 cleavage stage human-arrested embryos, the majority showed signs of apoptosis including TUNEL-labeling of the nuclei. In addition, agents such as staurosporine do induce apoptosis very early during development (Weil et al. 1996, Matwee et al. 2000). In mammalian development, apoptosis normally occurs at the blastocyst stage and arises in both the ICM and TE (Mohr & Trounson 1982, Handyside & Hunter 1986, Brison & Schultz 1997, Jurisicova et al. 1998). During this stage of development, one purpose of programmed cell death may be to eliminate cells with TE potential from the ICM (Pierce et al. 1989). There are many insults that induce apoptosis in the preimplantation embryo, one of which is glucose deprivation. Previously it was shown that the down-regulation of certain glucose transporters during preimplantation development results in increased apoptosis at the blastocyst stage. Specifically the down-regulation of either GLUT1 via high glucose concentrations in vitro or GLUT1 and GLUT8 utilizing antisense oligonucleotides results in increased apoptosis at the blastocyst stage (Chi et al. 2000a, Pinto et al. 2002a). In the case of GLUT8, down-regulation via antisense oligonucleotides leads to increased fetal resorption rates when the blastocysts were transferred into pseudo-pregnant recipient mice. Thus down-regulation of glucose transporter expression resulting in altered intraembryonic glucose concentrations leads to the induction of apoptosis in blastocysts.

Women with diabetes mellitus are at a higher risk for spontaneous abortions and fetal malformations (Greene et al. 1989, Casson et al. 1997, Hawthorne et al. 1997). Studies have established that an insult to the preimplantation embryo, triggered by maternal hyperglycemia, has long lasting detrimental effects on development. The hypothesis is that the decrease in glucose transport resulting from the hyperglycemia-induced down-regulation of the GLUTs, results in apoptosis of key progenitor cells or that this apoptosis and metabolic changes adversely affect the differentiation of the remaining cells. Either or both of these events then lead to either increased pregnancy resorptions or malformations. This hypothesis has been substantiated by two recent studies. First, Heilig et al. (2003) developed a transgenic mouse over expressing GLUT1 antisense. The homozygote GLUT1AS fetuses did not survive, and demonstrated a 7-fold higher stillborn rate than controls. Embryonic GLUT1 deficiency was associated with growth retardation (31.1%) and major malformations (35.3%) consistent with those seen in infants of diabetic women including caudal regression, anencephaly, microphthalmia, and micrognathia. Examination of homozygote embryos at the blastocyst stage revealed increased apoptosis and decreased glucose transport, consistent with the embryos derived from diabetic mice, suggesting that the decrease in glucose transport and resulting apoptosis may be responsible for the developmental abnormalities seen in the GLUT1AS model. Second, in recent studies fetuses that developed from diabetic 1-cell embryos, which were transferred into normal pseudo-pregnant recipient female mice were consistently and significantly smaller than controls (K Moley, unpublished observations). Importantly, these fetuses have approximately a 10% malformation rate, whereas no malformations were detected in control fetuses. The malformations detected in the diabetic embryos consisted of skeletal anomalies and they demonstrated delayed neural tissue development. Similarly, blastocysts recovered from diabetic mice and then transferred into normal recipient females develop malformations, whereas control blastocysts do not. The control blastocysts gave rise to fetuses that had 0% malformations, were on average 1.2 cm in length, and had an overall resorption rate of 21%. In contrast, blastocysts derived from diabetic mice gave rise to fetuses that had a 33% malformation rate, were on average 0.9 cm in length, and had an overall resorption rate of 52%. These data establish that the preimplantation period in mammalian development is a critical stage and that a hyperglycemic insult incurred during this period alone can have long lasting detrimental effects on embryo survival and development (K Moley, unpublished observations). Other studies have also demonstrated that apoptosis induced at this early stage leads to abnormal development and poor pregnancy outcome (Chi et al. 2000a, Pinto et al. 2002b, Heilig et al. 2003).

PI3-K and glucose metabolism

One hypothesis for the etiology of diabetes-associated malformations is hyperglycemia-induced apoptosis (Pampfer et al. 1997, Phelan et al. 1997, Moley et al. 1998a). Exaggerated apoptosis during the preimplantation period may result in fetal resorption or malformation due to the loss of key progenitor cells. Using a mouse diabetes model it was shown that hyperglycemia results in decreased glucose transport at the blastocyst stage, that corresponds with decreased GLUT1 protein expression (Moley et al. 1998b). As a consequence of glucose transporter down-regulation, these blastocysts have lower intraembryonic glucose concentrations. This decrease in
glucose utilization by the blastocyst occurs concurrently with increased apoptosis in the embryo (Chi et al. 2000a). Similar results have been found in other cell systems, including retinal pericytes (Mandarino et al. 1994, Li et al. 1998) where hyperglycemia-induced apoptosis of these cells is thought to play a role in the development of diabetic retinopathy. The cell death that occurs in the blastocysts requires both p53 and Bax (Moley et al. 1998a, Chi et al. 2000a, Keim et al. 2001). Embryos derived from diabetic Bax-deficient mice demonstrated decreased glucose uptake but no apoptosis (Chi et al. 2000a). In addition, the diabetic Bax<sup>−/−</sup> embryos exhibit a decreased resorption and malformation rate as compared with controls. This data suggests that hyperglycemia results in decreased glucose transport and that this event may trigger apoptosis in the murine blastocyst leading to poor pregnancy outcome. In vitro studies in which 2-cell embryos were cultured to the blastocyst stage in the presence of high glucose concentrations also resulted in decreased glucose transporter expression, decreased glucose uptake, and increased apoptosis (Moley et al. 1998a, Chi et al. 2000a). Therefore, inhibition of glucose uptake and thus metabolism via the down-regulation of specific glucose transporters is linked to the induction of apoptosis in murine blastocysts. These data imply that glucose transport and the maintenance of optimal intraembryonic glucose concentrations are crucial for preimplantation embryo development and survival.

Some of the first direct in vivo evidence demonstrating the importance of specific PI3-K subunits in the regulation of insulin sensitivity and glucose homeostasis came from genetic deletion studies. Mice deficient in the p85α subunit of PI3-K show increased insulin sensitivity and hypoglycemia (Terauchi et al. 1999). Insulin-stimulation of muscle and adipocytes derived from the null mice results in increased GLUT4 translocation to the plasma membrane with a corresponding increase in glucose transport. In addition, heterozygous deletion of p85α increases insulin sensitivity and glucose homeostasis in mice that are insulin-resistant due to heterozygous deletion of the insulin receptor or insulin receptor substrate-1 (IRS-1) (Mauvais-Jarvis et al. 2001). These data are surprising and several hypotheses have been put forward to explain this result. It has been suggested that the p85 subunit has negative regulatory effects on the p110 subunit either through competing with the p85/p110 heterodimer for receptor binding sites (Yu et al. 1998) or by inhibiting p110 activation by Ras (Chan et al. 2002, Jimenez et al. 2002). It is thought that insulin sensitivity is controlled by an equilibrium between the p85 and p110 subunits (Brachmann et al. 2005). Other studies have shown that p85β-null mice have enhanced insulin sensitivity and are both hypoinsulinemic and hypoglycemic (Ueki et al. 2002) while the p50α/p55α double knockout mice also display increased insulin sensitivity and insulin-stimulated glucose uptake in muscle and adipocytes (Chen et al. 2004). Finally, mice lacking p85α/p50α/p55α die a few days after birth and are hypoglycemic with decreased insulin levels and improved glucose tolerance (Fruman et al. 2000). Taken together these data suggest that the regulatory subunits of PI3-K play an essential role in insulin signaling and glucose metabolism.

A recent study conducted in murine blastocysts has suggested that PI3-K activity is critical for glucose uptake and metabolism during the preimplantation period. Similar to what was previously found in lymphocytes deprived of exogenous growth factors (Whetton et al. 1984, Kan et al. 1994, Rathmell et al. 2000, Vander Heiden et al. 2001) inhibition of the PI3-K/Akt pathway in murine blastocysts using LY-294002, resulted in decreased cell surface expression of GLUT1 with a corresponding decrease in 2-deoxyglucose uptake (Fig. 4) (Riley et al. 2005a). Along with the decrease in glucose utilization, the blastocysts displayed increased levels of apoptosis. Thus one mechanism by which PI3-K may promote embryo survival is through the maintenance of glucose uptake by the regulation of glucose transporter expression at the cell surface. A second mechanism by which PI3-K may regulate glycolysis is through its effects on the activity of glycolytic enzymes. Inhibition of the PI3-K pathway using LY-294002 resulted in decreased hexokinase activity at the blastocyst stage. Hexokinase is the first enzyme involved in the glycolytic pathway. It converts intracellular glucose to glucose-6-phosphate and thus a decrease in hexokinase activity may have a large effect on the overall rate of glucose utilization. It was previously shown in lymphocytes that the ability of activated Akt to inhibit apoptosis requires the presence of glucose and is linked to its metabolism (Plas et al. 2001, Rathmell et al. 2003). A separate study demonstrated the anti-apoptotic activity of Akt requires the first committed step of glucose metabolism that is catalyzed by hexokinase (Gottlob et al. 2001). Finally, Akt was shown to increase hexokinase activity (Gottlob et al. 2001, Rathmell et al. 2003). Taken together these data suggest that the PI3-K/Akt pathway is critical for glucose metabolism in the preimplantation embryo via its ability to regulate GLUT1 expression at the plasma membrane and thus glucose uptake by blastocysts as well as the activity of a key glycolytic enzyme.

The inhibition of glucose metabolism is in large part sufficient to explain the physiologic outcomes of inhibiting the PI3-K pathway in preimplantation embryos. We have demonstrated that iodoacetate, a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) inhibitor, induces apoptosis in both blastocysts and TS cells (Fig. 5A) (Riley et al. 2005a). GAPDH is a glycolytic enzyme which catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3 bisphosphoglycerate resulting in the production of NADH. These findings suggest that the maintenance of glycolysis is important for embryo survival as the inhibition of this pathway results in the induction of cell death. Importantly, a significant increase in the number of fetal resorptions was seen in embryos derived from iodoacetate-treated blastocysts as compared with controls.
Therefore, the maintenance of glycolysis at the blastocyst stage is crucial for subsequent developmental success. Importantly, one mechanism by which PI3-K promotes cell survival and reproductive success in preimplantation embryos is through the maintenance of glucose metabolism. A previous study showed that the inhibition of glycolysis later in development also has deleterious effects on the fetus.

(Fig. 4) Inhibition of PI3-K results in decreased GLUT1 expression at the plasma membrane. Reproduced from Riley et al. 2005a. Blastocysts were recovered and cultured in vitro in the presence of DMSO (vehicle control) or 250 μM LY-294002. The embryos were subjected to the TUNEL assay (red), immunofluorescent staining for GLUT1 or GLUT3 (green), and nuclear staining (blue).

(Fig. 5) Inhibition of GAPDH results in the induction of apoptosis in blastocysts and an increased frequency of fetal resorptions. Reproduced from Riley et al. 2005a. (A) Blastocysts were recovered and cultured in vitro in the presence of HTF media alone or increasing concentrations of the GAPDH inhibitor iodoacetate. The TUNEL assay was performed and the apoptotic nuclei are depicted in red. Embryos were counterstained with the nuclear dye TO-PRO-3 iodide as shown in blue. (B) Blastocysts were recovered and then cultured in vitro for 24 h in media containing either HTF alone or 2.5 μM iodoacetate. Unhatched blastocysts were then transferred back into the uterine horn of pseudopregnant female recipient mice at 2.5 dpc. This panel shows representative uterine horns derived from mice into which blastocysts cultured in either control media or iodoacetate were transferred.
Wentzel et al. (2003) demonstrated that the inhibition of GAPDH activity by iodoacetate in gestational day 11 rat embryos, results in increased malformation rates as well as decreased size, somite number, and DNA and protein content. Thus the inhibition of glycolysis has harmful effects on both pre- and post-implantation embryo development.

**Conclusion**

A growing body of evidence suggests that the PI3-K pathway plays a critical role in embryo survival. In addition PI3-K activity regulates glucose utilization in the preimplantation blastocyst. The ability of PI3-K to promote embryo survival and metabolism depends on its ability to maintain glucose uptake and the activity of at least one glycolytic enzyme (Fig. 6). It will be important to determine whether this key survival pathway is inhibited during the preimplantation period in maternal disease states such as diabetes and insulin resistance that are known to affect glucose utilization in the preimplantation embryo and have adverse effects on pregnancy outcome.

**References**


**Acknowledgements**

This work is supported by a Juvenile Diabetes Research Foundation Fellowship to J K R and by HD40390 and DK0070351 from the National Institutes of Health to K H M. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

![Figure 6](image)

**Figure 6** PI3-K activity regulates glucose utilization in the preimplantation blastocyst. The ability of PI3-K to promote embryo survival and metabolism depends in part on its ability to maintain glucose uptake. PI3-K activity is required to retain the facilitative glucose transporter GLUT1 at the plasma membrane. It is thought that Akt is involved in the mechanism by which PI3-K promotes GLUT1 cell surface expression. In addition the PI3-K pathway is required for the optimal activity of at least one glycolytic enzyme in blastocysts.
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Received 3 November 2005
First decision 17 November 2005
Revised manuscript received 9 January 2006
Accepted 19 January 2006

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