PI3-KINASE ACTIVITY IS CRITICAL FOR GLUCOSE METABOLISM AND EMBRYO SURVIVAL IN MURINE BLASTOCYSTS†
Joan K. Riley,1 Mary O. Carayannopoulos,3,5 Amanda H. Wyman,3 Maggie Chi,3 and Kelle H. Moley2,3,4
From the Departments of Obstetrics and Gynecology,3 Cell Biology and Physiology4 and Pediatrics5
Washington University School of Medicine, St. Louis, Missouri, 63110
Running Title: PI3-K regulates glucose metabolism and embryo survival
Address correspondence to: Kelle H. Moley, Department of Obstetrics and Gynecology, Box 8064, Washington University School of Medicine, 4911 Barnes-Jewish Hospital Plaza, St. Louis, Missouri, 63110. Tel. 314-362-1997; Fax. 314-747-4150; E-mail: moleyk@wustl.edu

The phosphatidylinositol 3-kinase (PI3-K) signal transduction pathway is a well-known mediator of cell growth, proliferation, and survival signals. While the expression and function of this pathway has been documented during mammalian development, evidence demonstrating the physiologic importance of this pathway in murine preimplantation embryos is beginning to emerge. This study demonstrates that inhibition of the PI3-K pathway leads to the induction of apoptosis in both murine blastocysts and trophoblast stem (TS) cells. The apoptosis induced in both model systems correlates with a decrease in the expression of the glucose transporter GLUT1 at the plasma membrane. In addition, blastocysts cultured in the presence of the PI3-K inhibitor LY-294002 display a decrease in both 2-deoxyglucose uptake and hexokinase activity as compared to control blastocysts. To determine the impact of PI3-K inhibition on pregnancy outcome, embryo transfer experiments were performed. Blastocysts cultured in the presence of LY-294002 demonstrate a dramatic increase in fetal resorptions as compared to control embryos. Finally, we demonstrate that impairment of glucose metabolism via iodoacetate (IA), a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) inhibitor, is sufficient to induce apoptosis in both blastocysts and TS cells. Moreover, blastocysts treated with IA result in poor pregnancy outcome as determined by embryo transfer experiments. Taken together these data demonstrate the critical importance of the PI3-K pathway in preimplantation embryo survival and pregnancy outcome and further emphasize the importance of glucose utilization and metabolism in cell survival pathways.

The blastocyst stage of murine preimplantation development occurs approximately four days post-fertilization. At this stage of embryonic development the first cell differentiation step has occurred. The blastocyst is comprised of the epithelial trophectoderm (TE), which is the layer of cells that develops into the placenta, and the inner cell mass (ICM), which consists of the pluripotent cells that gives rise to the embryo proper. Prior to implantation, the developing embryo is dependent on signals generated by growth factors that are either made by the embryo itself or are present in the maternal environment. These growth factors are known to regulate cellular proliferation and differentiation during mammalian preimplantation development (1,2). Importantly, the preimplantation embryo expresses a number of growth factor receptors known to activate the PI3-K pathway including the insulin and IGF-I receptors (3,4).

PI3-K is a lipid kinase that phosphorylates the D-3 position on the inositol ring in phosphoinositides (5-7). It is a heterodimeric enzyme that consists of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. A number of extracellular signals activate PI3-K including insulin and other growth factors. Insulin and IGF-I have been
shown to have both mitogenic and antiapoptotic effects on mammalian preimplantation embryos (8-15). Insulin stimulation results in the recruitment of PI3-K to the plasma membrane where it subsequently phosphorylates its lipid substrate (16). The phosphorylation of phosphoinositides by PI3-K results in the recruitment of the serine-threonine kinase Akt to the plasma membrane. Once at the membrane Akt itself becomes phosphorylated and thus activated and can go on to phosphorylate downstream targets including both pro and antiapoptotic proteins (17). In general it is believed that Akt is the primary mediator of the antiapoptotic signal generated via the PI3-K pathway (18-21).

In addition to the direct phosphorylation of either pro or antiapoptotic proteins, the PI3-K/Akt pathway is thought to play a role in cell survival via its effects on glucose uptake and metabolism (22). Akt has been shown to effect glucose uptake and glycolysis by inducing the translocation of both facilitative glucose transporters, GLUT1 and GLUT4, to the plasma membrane (23,24). More recently, active forms of Akt have been shown to stimulate the activity of hexokinase, the first enzyme in the glycolytic pathway (25,26). Thus in some cell types the PI3-K/Akt pathway regulates glycolysis by controlling both glucose entry into the cell via the expression of glucose transporters at the cell surface and by regulating the activity of enzymes involved in the glycolytic pathway.

The PI3-K/Akt pathway has been examined during different developmental processes however relatively little is known about this pathway during the preimplantation period. This is a critical stage during development as several studies have suggested that perturbations in glucose and amino acid metabolism may have long lasting effects on later development (27-31). Previously we demonstrated that the 85 kDa regulatory subunit and the 110 kDa catalytic subunit of PI3-K as well as the serine-threonine kinase Akt are expressed from the 1-cell through the blastocyst stage of murine preimplantation embryo development (32). These proteins are localized predominantly at the cell surface from the 1-cell through the morula stage. At a blastocyst stage, both PI3-K and Akt exhibit a largely apical staining pattern suggesting that these proteins are expressed on the apical surface of the polarized trophoderm cells. Interestingly, we found that Akt is phosphorylated throughout murine preimplantation development implying that this pathway is being continuously activated by endogenous growth factors during this early developmental period.

Prior studies demonstrated the importance of the PI3-K pathway for cell survival during the preimplantation period. Deletion of the p110β subunit of PI3-K leads to early embryonic lethality (33). There is a deficit in the number of homozygous knockout embryos detected at E3.5 (blastocyst stage) implying that this protein is important during early development. In addition studies have shown that the inhibition of PI3-K leads to decreased numbers of embryos that develop to the morula and blastocyst stage when cultured in vitro and to the induction of apoptosis during this developmental period (34,35).

Previously we showed that inhibition of PI3-K activity also has significant effects on the normal physiology of the blastocyst. Specifically, inhibition of this pathway results in decreased insulin stimulated glucose uptake (32). Moreover, inhibiting PI3-K activity results in a significant delay in blastocyst hatching, a developmental step required for implantation. We also determined that trophoblast stem cells express the functional proteins of the PI3-K pathway making this a useful in vitro model to study this signaling cascade.

To date however there is no data regarding the physiologic and mechanistic impact of inhibiting the PI3-K pathway on glucose uptake and metabolism in preimplantation embryos and the effect on later developmental potential. Therefore in this study the physiologic importance of this pathway for glucose metabolism and embryo survival in the murine preimplantation blastocyst and in a TS cell line is examined. These experiments demonstrate that the PI3-K pathway inhibits apoptosis in both murine blastocysts and trophoblast stem cells. Inhibition of this pathway leads to the induction of blastocysts and trophoblast stem cells. Inhibition of this pathway leads to the induction of apoptosis in both murine blastocysts and trophoblast stem cells. Inhibition of this pathway leads to the induction of apoptosis in both murine blastocysts and trophoblast stem cells.
Notably, inhibition of the PI3-K pathway at the blastocyst stage results in increased fetal resorptions as well; suggesting that short-term inhibition at the blastocyst stage results in later pregnancy failure. We demonstrate that impairment of glucose metabolism alone is sufficient to induce apoptosis in both blastocysts and TS cells. Moreover, the inhibition of glycolysis at the blastocysts stage results in poor pregnancy outcome. Thus, elucidating the functional importance of the PI3-K pathway in maintaining glucose metabolism during preimplantation development is critical for understanding the mechanisms involved in preimplantation embryo survival and reproductive success.

Materials and Methods

**Embryos Recovery and Culture** - Embryos were recovered as previously described (36). In short, three-week-old female mice (B6 x SJL F1, Jackson Laboratories; Bar Harbor, ME) were given free access to food and water and were maintained on a 12-hour light/dark cycle. Female mice were superovulated with an intraperitoneal injection of 10 International Units (IU)/animal of pregnant mare serum gonadotropin (PMSG, Sigma; St. Louis, MO) followed 48 hours later by 10 IU/animal of human chorionic gonadotropin (hCG, Sigma; St. Louis, MO). Female mice were mated with males of proven fertility overnight following the hCG injection. Mating was confirmed by identification of a vaginal plug. Mice were sacrificed 96 hours post hCG injection to recover embryos at the blastocyst stage. Embryos were recovered by flushing dissected uterine horns and ostia with human tubal fluid medium (HTF, Irvine Scientific; Santa Ana, CA) containing 0.25% BSA (Bovine serum albumin fraction V, Sigma; St. Louis, MO). Female mice were mated with males of proven fertility overnight following the hCG injection. Mating was confirmed by identification of a vaginal plug.

Mice were sacrificed 96 hours post hCG injection to recover embryos at the blastocyst stage. Embryos were recovered by flushing dissected uterine horns and ostia with human tubal fluid medium (HTF, Irvine Scientific; Santa Ana, CA) containing 0.25% BSA (Bovine serum albumin fraction V, Sigma; St. Louis, MO). To determine the effect of the GAPDH inhibitor sodium iodoacetate (Sigma; St. Louis, MO) on apoptosis induction blastocysts were harvested 96 hours post hCG injection and subsequently incubated in vitro for an additional 30 hours in HTF media alone or HTF supplemented with 2.5, 5 or 7 µM iodoacetate. These experiments were conducted at least three times each. All procedures described above were reviewed and approved by the animal studies committee at Washington University and were performed in accordance with IACUC approval.

**Cells** - Primary TS cells were a generous gift from Dr. Leonidas Carayannopoulos (Washington University, St. Louis MO) and were generated from C57/B6 mice following published protocols (37). To confirm the trophoblast lineage of this cell line, several genetic markers were analyzed. These markers included two TS specific genes, Errβ and Hand1, which were both expressed in this cell line, one embryonic stem (ES) cell specific marker, Oct3/4, which was not expressed in this cell line and one mesoderm specific gene, T Brachyury, which was not expressed in this cell line. TS cells were maintained in the absence of mouse embryonic fibroblast (MEF) feeder layers, cultured instead in the presence of MEF conditioned media (MEF-CM). Briefly cells were grown in 70% feeder-conditioned medium and 30% TS medium supplemented with FGF-4 (25 ng/ml) (Sigma; St. Louis, MO) and heparin (1 µg/ml) (Sigma; St. Louis, MO). TS medium consisted of 20% fetal bovine serum (HyClone; Logan, UT), 1 mM sodium pyruvate (Cambrex Bio Science; Walkersville, MD), 100 µM β-mercaptoethanol (Sigma; St. Louis, MO), 2 mM L-glutamine (Cambrex Bio Science; Walkersville, MD) and 50 µg/ml penicillin/streptomycin (Cambrex Bio Science; Walkersville, MD) in RPMI-1640 (Cambrex Bio Science; Walkersville, MD). To generate feeder-conditioned medium mouse embryo fibroblasts were irradiated (5000 Rads) and plated in 10 cm dishes at a density of 2 x 10⁵ cells/ml in TS medium (10 ml/plate). The cells were cultured for at least 72 hours and the media was subsequently collected. Floating cells were...
removed from the cultured medium via centrifugation and the medium was then 0.22 µm filtered and stored at -20°C. TS cells were cultured in either DMSO or 150 µM LY-294002 for 0, 6, 12 and 24 hours or HTF media alone or 20 µM IA for 6, 12 and 24 hours and the percentage of apoptotic cells was determined using the TUNEL assay followed by flow cytometry. In experiments that examined the effect of LY-294002 on GLUT1 surface expression TS cells were cultured in either DMSO or 150 µM LY-294002 for 12 hours and plasma membrane fractions were subsequently isolated and analyzed by Western blot. These experiments were conducted at least three times each and the results shown are from one representative experiment.

**TUNEL Assay -** Apoptosis was assayed using terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) as previously described (38). Embryos were fixed in 3% paraformaldehyde (PFA) (Sigma; St. Louis, MO) for 20 minutes and then permeabilized in 0.1% Tween-20 (Sigma; St. Louis, MO) for 30 minutes. TS cells were fixed in 2% PFA for 1 hour and then permeabilized in 0.1% Triton X-100 (Sigma; St. Louis, MO) for 2 minutes. Apoptosis was assessed using the In Situ Cell Death Detection Kit, TMR (Roche; Basel, Switzerland) according to the manufacturer’s protocol. After the TUNEL assay was performed the nuclei of the embryos were stained using 4 µM To-Pro-3-iodide (Molecular Probes; Eugene, OR) for 20 minutes. In experiments in which the embryos were both immunofluorescently labeled and the TUNEL assay was performed the TUNEL assay was performed first followed by antibody staining of the embryos. A Z-series consisting of 5 sections was taken for each embryo using a Nikon C1 laser-scanning confocal microscope. The total number of nuclei and apoptotic nuclei per embryo were quantitated manually for each Z-series. The sum of the apoptotic nuclei and total nuclei within each treatment group was then utilized to calculate the percentage of TUNEL positive nuclei per embryo. This experiment was performed at least three times with a minimum of 5 embryos per group.

**Flow Cytometry -** TS cells were seeded at a density of 2.5 x 10^5 per 10 cm dish. The cells were cultured in the presence of either DMSO or 150 µM LY-294002 or HTF media alone or 20 µM IA for 6, 12 or 24 hours. The TS cells were subsequently harvested, washed twice in PBS and the TUNEL assay was performed according to the manufacturer’s protocol. The cells were analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson; Mountain View, CA). An arbitrary gate was drawn and the percentage of cells within the gate was determined using Cell Quest software (Becton Dickinson; Mountain View, CA). This experiment was performed at least three times and the results are expressed as the mean ± SEM.

**2-Deoxyglucose Uptake and Hexokinase Assay -** Glucose uptake into single blastocysts was measured using a nonradioactive microanalytic procedure described previously (36,39). In short, blastocysts after 30 hours in culture in the presence of DMSO or 250 µM LY-294002 were incubated at 25°C in 200 µM 2-deoxyglucose (DG) for 15 minutes, washed in DG- and BSA-free buffer for 1 minute, and then were quick-frozen on a glass slide. After freeze-drying overnight, the embryos were extracted in microliter volumes under oil and assayed for DG as described previously. The final measurements are expressed as picomoles per embryo per 15 minutes. Experiments were performed in triplicate on 20 individual embryos per group for each experiment and the results are expressed as the mean ± SEM. For the hexokinase measurements, embryos were treated as described above and hexokinase levels were measured on individual blastocysts as described previously (40). Hexokinase activity is expressed as picomoles per embryo per hour. This experiment was performed at least three times with a minimum of 20 embryos per treatment group and the results are expressed as the mean ± SEM.

**Immunofluorescent Staining -** Immunofluorescence staining techniques have been described for embryo preparations previously (36). All labeling was performed in microdroplets. Embryos were fixed in 3% PFA (Sigma; St. Louis, MO) for 20 minutes and then
permeabilized in 0.1% Tween-20 (Sigma; St. Louis, MO) for 30 minutes. The embryos were subsequently blocked for 1 hour with 20% normal goat serum (Pierce; Rockford, IL) in phosphate-buffered saline containing 2% bovine serum albumin (PBS/BSA). Embryos were then washed three times for 10 minutes each in PBS/BSA and then incubated for 40 minutes in 20 μg/ml of one of the following antibodies: anti-GLUT1 (kindly provided by Dr. Michael Mueckler, Washington University, St. Louis, MO), or anti-GLUT3 (kindly provided by Dr. Sherin Devaskar, Los Angeles, CA). The embryos were then washed three times for 10 minutes each in PBS/BSA and incubated with the secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes; Eugene, OR) at a concentration of 2 μg/ml for 30 minutes followed by 4 μM To-Pro-3-iodide (Molecular Probes; Eugene, OR), a nuclear stain, for 20 minutes. Finally the embryos were washed three times for 10 minutes each in PBS and mounted in drops of Vectashield (Vector Laboratories; Burlingame, CA) under a coverslip. Fluorescence was detected with a Nikon C1 laser-scanning confocal microscope. Confocal images were taken at x 63 magnification. These experiments were performed three times with at least 5 embryos per group for each experiment.

**Plasma Membrane Preparation -** TS cells were seeded at a density of 2.5 x 10⁶ per 10 cm dish. The cells were cultured in the presence of either DMSO or 150 μM LY-294002 for 12 hours. The TS cells were subsequently harvested, washed once in homogenization buffer (20 mM Tris pH 7.4, 1 mM EDTA, 255 mM sucrose) and then resuspended in homogenization buffer containing protease inhibitors (HB+PI) (EMD Biosciences; La Jolla, CA). The cells were lysed using a dounce homogenizer and then centrifuged at 1,750 rpm for 10 minutes. The pellet was then resuspended and centrifuged and the pooled supernatant was subsequently centrifuged at 10,000 rpm for 15 minutes. The pellet was resuspended in 1 ml HB+PI and homogenized using a dounce. The homogenate was overlaid on a 38.5% sucrose gradient and centrifuged at 33,000 rpm for 1 hour. The interface containing the plasma membrane fraction was collected and diluted with 10 mM Tris pH 7.4. The membrane fraction was centrifuged at 22,000 rpm for 1 hour. The plasma membrane pellet was resuspended in 10 mM Tris and the protein level was quantitated. Protein contents were determined by BCA protein assay (Pierce; Rockford, IL). 2 μg of protein was loaded per lane on a 12% SDS-PAGE gel and analyzed by Western blot using an anti-GLUT1 antibody. The membrane was simultaneously probed with an anti-actin antibody (Chemicon; Temecula, CA) to normalize for protein loading.

**Embryo Transfers -** Blastocysts were harvested as described above and then cultured in vitro for 24 hours in HTF/BSA containing either DMSO or 250 μM LY-294002 or HTF media alone or 2.5 μM IA. Unhatched blastocysts were then transferred back into the uterine horn of pseudopregnant female recipient mice at 2.5 dpc (41). 8-10 blastocysts were transferred to a single uterine horn, resulting in 16-20 embryos per recipient. The mice were sacrificed and the embryos examined at E14.5. Four independent experiments were performed. A total of 7 control mice with 72 implantation sites and 8 LY-294002 mice with 121 implantation sites were analyzed. For the IA transfer experiments four independent experiments were performed. A total of 7 control mice with 87 implantation sites and 7 IA mice with 78 implantation sites were analyzed. The percentage of fetal resorption per mouse was calculated by dividing the number of resorptions by the total number of implantation sites. The per mouse resorption percentage was then averaged for the control or the treated group over all four experiments. The results are expressed as the mean ± SEM.

**Statistical Analysis -** Differences between DMSO and LY-294002 and control and IA induced apoptosis in blastocysts were analyzed using ANOVA with a Fisher’s post-hoc test while differences in TS cell apoptosis were analyzed using ANOVA with a Bonferroni/Dunn post-hoc test (Statview 4.5, Abacus Concepts, Berkeley, CA). Differences in 2-deoxyglucose uptake, hexokinase activity, and fetal resorption percentages between DMSO and LY-294002 treated blastocysts were analyzed using an unpaired student’s t-test. Differences in fetal resorption percentages for control vs IA treated blastocysts were analyzed using an unpaired...
RESULTS

Previously we and others demonstrated that the PI3-K pathway is present and functional during murine preimplantation development (32,34,35,42); however, there is still relatively little data regarding the physiologic ramifications of inhibiting this pathway in preimplantation embryos. Due to the connection between the PI3-K signal transduction pathway, growth factors, and cell survival in other systems (18,43), we first investigated whether inhibition of PI3-K results in the induction of apoptosis. Blastocysts were cultured in vitro in either DMSO (vehicle control) or increasing concentrations of the PI3-K inhibitor LY-294002 and the TUNEL assay was performed. A dose dependent increase in the percentage of apoptotic nuclei per blastocyst was seen (Fig. 1A). The number of TUNEL positive nuclei per embryo was quantitated and found to be 4.0% (± 0.9) and 18.6% (± 3.1) (p<0.001) at the 250 µM and 500 µM doses of LY-294002 respectively (Fig. 1B). The 750 µM, and 1 mM doses of LY-294002 resulted in amounts of apoptosis that were too numerous to quantify. These findings suggest that the PI3-K pathway is important for embryo survival as the inhibition of this pathway results in the induction of apoptosis at the blastocyst stage.

Both PI3-K and Akt are largely expressed on the apical surface of trophectoderm cells at the blastocyst stage (32). Since TS cells are derived from the trophectoderm of murine blastocysts these cells are an ideal in vitro correlate to confirm that inhibition of this pathway results in apoptosis in these embryonic cells. TS cells were cultured in either DMSO or 150 µM LY-294002 for 0, 6, 12, or 24 hours and the TUNEL assay performed. The TS cells were subsequently analyzed by flow cytometry. TS cells began to undergo apoptosis after 12 hours of LY-294002 treatment (Fig. 2A). The amount of apoptosis induced was further increased at the 24-hour time point. To quantify the relative amounts of apoptosis induced by LY-294002 treatment an arbitrary gate was drawn (M1) and the percentage of cells within the gate was determined using Cell Quest software. Shown in Fig. 2B is the percentage of cells falling in the M1 gate for each treatment group. On average 37.1% (± 2.2) (p<0.001) of the cells at the 12-hour time point fell within the gate. This number increased to 64.3% (± 5.1) (p<0.0001) at the 24-hour time point. Thus this experiment confirms the blastocyst study by demonstrating that inhibition of the PI3-K pathway in TS cells results in the induction of cell death.

In accordance with studies showing a relationship between PI3-K/Akt signaling and glucose utilization, the next studies focused on determining whether a change in glucose transporter expression at the plasma membrane occurs in response to PI3-K inhibition. Glucose can enter cells via a family of facilitative glucose transporters known as GLUTs (44,45). Although the mechanism by which glucose enters the mammalian preimplantation embryo is not completely understood it is known that at the blastocyst stage the glucose transporter GLUT3 is expressed on the apical surface of polarized trophectoderm cells and thus appears as a ring surrounding the blastocyst on immunofluorescent confocal images (46). In contrast, GLUT1 is expressed on the basolateral surface of trophectoderm and on the plasma membrane of the nonpolarized inner cell mass (46). This pattern of expression leads to a honeycomb-like staining pattern (27). To determine whether PI3-K inhibition leads to decreased plasma membrane expression of GLUT1 blastocysts were cultured in vitro in either DMSO or 250 µM LY-294002 and then the TUNEL assay was performed followed by immunofluorescent staining using antibodies to GLUT1 or GLUT3. Finally, the embryos were stained with a nuclear dye. Blastocysts cultured in DMSO showed few TUNEL positive nuclei and displayed plasma membrane staining of GLUT1 (Fig. 3A). In contrast, blastocysts cultured in LY-294002 showed an increase in TUNEL positive nuclei and a lack of GLUT1 staining at the plasma membrane although cytoplasmic staining of the protein was detected. The relocalization of the transporter appeared to be specific to GLUT1 as the apical staining

student’s t-test. Results are expressed as means ± standard error of at least three separate experiments. Differences were considered significant at p<0.05.
pattern of GLUT3 was unaltered when the embryos were cultured in the presence of the PI3-K inhibitor. Similar results were found when TS cells were treated with LY-294002. Shown in Fig. 3B is a western blot analysis of plasma membrane fractions isolated from TS cells cultured in either DMSO or 150 µM LY-294002. GLUT1 is highly glycosylated and thus appears as a diffuse band. Plasma membranes isolated from LY-294002 treated TS cells contained less GLUT1 protein than cells cultured in DMSO. Western blot analysis of whole cell lysates did not reveal any differences in GLUT1 levels between control and LY-294002 treated TS cells (data not shown). Thus inhibition of the PI3-K pathway leads to decreased expression of the glucose transporter GLUT1 at the plasma membrane in both blastocysts and TS cells.

Given that inhibition of the PI3-K pathway leads to decreased plasma membrane expression of GLUT1, glucose uptake into blastocysts should also reflect a similar decrease. Nonradioactive 2-deoxyglucose uptake into single blastocysts was measured with a microfluorometric assay combined with enzymatic cycling reactions. As shown in Fig. 4, blastocysts cultured in the presence of LY-294002 showed an approximate 29% decrease in 2-deoxyglucose uptake as compared to blastocysts cultured in DMSO. 2-deoxyglucose concentrations in control blastocysts was on average 1.984 (±0.045) mmoles/kg wet weight/15 minutes as compared to 1.402 (±0.089) mmoles/kg wet weight/15 minutes for LY-294002 treated blastocysts (p<0.001). Not all of the blastocysts responded to the LY-294002 treatment at the 30-hour timepoint, thus only the data from the responders are displayed. Glucose uptake in nonresponders was not significantly different from controls (data not shown). This data shows that inhibition of the PI3-K pathway not only leads to decreased plasma membrane expression of GLUT1 but also to a corresponding decrease in glucose uptake in blastocysts.

Hexokinase is the first enzyme involved in the glycolytic pathway. It catalyzes the conversion of glucose to glucose-6-phosphate thus preventing glucose from exiting the cell via the bidirectional transporters (47). Therefore hexokinase activity affects the rate of glucose uptake. Prior studies have shown that the PI3-K pathway regulates hexokinase activity (25,26,48), therefore the effect of PI3-K inhibition on hexokinase activity in the blastocyst was examined. Blastocysts were cultured in either DMSO or LY-294002 and hexokinase activity in each individual blastocyst was measured. As depicted in Fig. 5 blastocysts treated with LY-294002 showed a 35% decrease in hexokinase activity as compared to control blastocysts. Hexokinase activity in control embryos was 93.2 (± 1.7) pmoles/embryo/hour as compared to 60.1 (± 1.9) pmoles/embryo/hour for LY-294002 treated embryos (p<0.001). Again, not all of the blastocysts responded to the LY-294002 treatment at the 30-hour timepoint and only the responders were analyzed. Hexokinase activity in nonresponders was not significantly different from controls (data not shown). This data demonstrates the PI3-K pathway plays a role in the maintenance of hexokinase activity in blastocysts.

Since inhibition of the PI3-K pathway in blastocysts results in increased apoptosis and deficiencies in both glucose uptake and metabolism, the next important question to be answered was whether inhibition of this pathway at this stage has adverse effects on pregnancy outcome. Blastocysts were cultured in vitro in either DMSO or 250 µM LY-294002 and then transferred into pseudopregnant female mice. The mice were sacrificed at E14.5 and the embryos examined. Shown in Fig. 6A are uterine horns derived from mice into which blastocysts cultured in either DMSO or LY-294002 were transferred. We found a significant increase in the number of fetal resorptions seen in embryos derived from LY-294002 treated blastocysts as compared to controls. The number of resorptions per number of implantation sites was quantitated and the resulting percentage of fetal resorptions is depicted in Fig. 6B. Control blastocysts cultured in DMSO resulted in 12.5% (± 2.0) fetal resorptions whereas blastocysts cultured in LY-294002 resulted in 88.7% (±3.3) fetal resorptions (p<0.0001). Therefore, activation of the PI3-K pathway is critical at the blastocyst stage of development as inhibition of this
pathway during this stage resulted in a striking increase in pregnancy loss.

Having demonstrated that the inhibition of the PI3-K pathway is critical for embryo survival and successful pregnancy outcome as well as the maintenance of glucose metabolism we next wanted to determine whether the inhibition of glucose metabolism alone was sufficient to explain the physiologic outcomes of inhibiting the PI3-K pathway in preimplantation embryos. To address this issue blastocysts were cultured in vitro in either media alone or increasing concentrations of the GAPDH inhibitor iodoacetate and the TUNEL assay was performed. GAPDH is a glycolytic enzyme which catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3 bisphosphoglycerate resulting in the production of NADH. Blastocysts cultured for 24 hours in 1 µM iodoacetate demonstrated a 80% decrease in GAPDH activity while those cultured in 5 µM iodoacetate showed a 90% decrease in GAPDH enzyme activity (data not shown). Inhibition of this enzyme with IA resulted in an increase in the percentage of apoptotic nuclei per blastocyst (Fig. 7A). The number of TUNEL positive nuclei per embryo was quantitated and found to be 0.6% (±0.3) and 4.1% (±1.7) at the 2.5 µM and 5 µM doses of IA respectively which were not statistically different from controls (Fig. 7B). The 7 µM dose of IA resulted in 32.8% (±4.5) (p<0.0001) apoptotic nuclei per embryo. These findings suggest that the maintenance of glycolysis is important for embryo survival as the inhibition of this pathway results in the induction of apoptosis at the blastocyst stage.

We next confirmed that inhibition of the PI3-K pathway results in apoptosis in TS cells as well as blastocysts. TS cells were cultured in either media alone or 20 µM IA for 6, 12, or 24 hours and the TUNEL assay performed. The TS cells were subsequently analyzed by flow cytometry. TS cells began to undergo apoptosis after 6 hours of IA treatment (Fig. 8A). The amount of apoptosis induced was further increased at the 12 and 24-hour time points. To quantify the relative amounts of apoptosis induced by IA treatment an arbitrary gate was drawn (M1) and the percentage of cells within the gate was determined. Shown in Fig. 8B is the percentage of cells falling in the M1 gate for each treatment group. On average 42.4% (±7.2) (p<0.002) of the cells at the 6-hour time point, 62.3% (± 0.5) (p<0.0001) of the cells at the 12-hour time point and 93.9% (± 2.2) (p<0.0001) at the 24-hour time point fell within the gate. Thus the inhibition of glycolysis leads to the induction of apoptosis in both blastocysts and TS cells.

Finally we established whether the inhibition of glycolysis at the blastocyst stage would lead to adverse pregnancy outcome similar to what was found using the PI3-K inhibitor LY-294002. Blastocysts were cultured in vitro in either media alone or 2.5 µM IA and then transferred into pseudopregnant female mice. Again, the mice were sacrificed at E14.5 and the embryos examined. Shown in Fig. 9A are uterine horns derived from mice into which blastocysts cultured in either media alone or IA were transferred. We found a significant increase in the number of fetal resorptions seen in embryos derived from IA treated blastocysts as compared to controls. The number of resorptions per number of implantation sites was quantitated and the resulting percentage of fetal resorptions is depicted in Fig. 9B. Control blastocysts resulted in 15.8% (±3.8) fetal resorptions whereas blastocysts cultured in IA resulted in 65.5% (±7.7) fetal resorptions (p<0.0001). 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.

DISCUSSION

At the blastocyst stage of murine preimplantation development the embryo is dependent on signals generated by growth factors that are either made by the embryo itself or are present in the maternal environment for survival. Prior studies have demonstrated that apoptosis induced at this early stage leads to abnormal development and poor pregnancy outcome (27,29,49). Growth factors such as insulin-like growth factor-I (IGF-1), IGF-II, and transforming growth factor-α (TGF-α) have
been shown to suppress apoptosis in embryos (8,9,11-13,15,50,51). Recently it was demonstrated that the mechanism by which TGF-α inhibits apoptosis in preimplantation embryos involves the PI3-K pathway (42). Many of the growth factors that suppress apoptosis are known to activate the PI3-K/Akt signal transduction pathway. Importantly, the preimplantation embryo expresses a number of growth factor receptors known to activate this pathway thus one mechanism of embryo survival may involve growth factor activation of the PI3-K cascade. The signal transduction pathways that inhibit apoptosis in preimplantation embryos are not well elucidated. Clarifying the functional significance of the PI3-K pathway during preimplantation development may improve our understanding of mechanisms involved in both reproductive success and failure.

In this study, we demonstrate that the PI3-K pathway plays a key role in embryo survival as inhibition of this pathway results in the induction of apoptosis in both blastocysts and TS cells. Complementary studies by Gross et al. demonstrated that suppression of apoptosis in embryonic stem (ES) cells, which are derived from the inner cell mass of blastocysts, requires serum growth factors that activate the PI3-K pathway (35). Inhibition of this pathway using LY-294002 resulted in the induction of apoptosis in both ES cells and intact murine blastocysts. A separate study conducted by Lu et al. demonstrated that both LY-294002 and wortmannin treatment result in a reduction in the number of zygotes developing to the morula and blastocyst stages in vitro (34). In addition the total number of cells within each blastocyst was significantly reduced and the number of fragmented nuclei was increased. Taken together these results are consistent with the idea that activation of the PI3-K pathway is required for early embryo survival. Previous studies however have not examined the mechanism by which inhibition of this pathway may lead to cell death or whether inhibition of this pathway at the blastocysts stage leads to poor pregnancy outcome.

To begin to elucidate the mechanism by which PI3-K promotes embryo survival we examined the effects of LY-294002 on glucose uptake and metabolism. Inhibition of PI3-K resulted in decreased GLUT1 expression at the plasma membrane of both blastocysts and TS cells. Moreover, we found that LY-294002 treatment resulted in decreased 2-deoxyglucose uptake which is consistent with a decrease in plasma membrane localization of GLUT1. Thus one mechanism by which PI3-K may promote embryo survival is through the maintenance of glucose uptake via regulation of glucose transporter expression at the cell surface. Interestingly, the biologic response seen in blastocysts treated with the PI3-K inhibitor mirrors that which occurs in embryos derived from diabetic mice. 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 (36). 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 (27). It will be important to elucidate whether inhibition of the PI3-K pathway plays a causal role in the development of diabetic embryopathy.

A second mechanism by which the PI3-K pathway regulates glycolysis in preimplantation embryos is through its effects on glycolytic enzyme activity. We found that inhibition of PI3-K results in decreased hexokinase activity. Previously it was shown that the ability of activated Akt to inhibit apoptosis requires the presence of glucose and is linked to its metabolism (26,52). Prior studies also demonstrated that the antiapoptotic activity of Akt requires only the first committed step of glucose metabolism which is catalyzed by hexokinase (25). Gottlob et al. also showed that overexpression of hexokinase was able to inhibit cytochrome C release and apoptosis. The authors hypothesize that Akt may increase the pairing of glucose metabolism to oxidative phosphorylation by increasing hexokinase-VDAC interaction at the outer mitochondrial membrane. While we did not examine the association of hexokinase with the mitochondria, the decrease in hexokinase activity alone may exert a large effect on the overall rate of glucose uptake.
Previously we and others demonstrated that murine preimplantation embryos express the p85 and p110 subunits of PI3-K (32,34,42). The antibodies used in our study were not specific for a given isoform rather they were antibodies that recognized all p85 or p110 isoforms. LY-294002 is a specific inhibitor of PI3-kinases and is likely inhibiting many if not all PI3-K isoforms in the preimplantation embryo. We have previously shown that this inhibitor blocks the function of PI3-K in early embryos, as insulin stimulated glucose uptake is completely inhibited in blastocysts treated with either LY-294002 or an additional PI3-K inhibitor, wortmannin (32).

The deletion of certain PI3-K subunits results in embryonic lethality. Mice deficient in the p110α subunit of PI3-K die in utero between E9.5 and E10.5. These embryos possess a proliferation defect (53) as well as multiple vascular defects (54). The fetal resorption phenotype we found in LY-294002 treated blastocysts is most similar to the phenotype seen in the p110α knockout mice in that these embryos implant but die by E14.5. Interestingly, inhibition of the PI3-K pathway for only 24 hours at the blastocyst stage results in a striking increase in the number of fetal resorptions. Similarly, when embryos were treated with LY-294002 for only 30 hours (throughout the zygote and 2-cell stage) and then cultured without the inhibitor for an additional 66 hours there was a significant reduction in the number of embryos reaching the blastocyst stage (34). Thus inhibition of the PI3-K pathway for short amounts of time has lasting effects on embryo development and survival.

To determine whether impairment of glucose metabolism similar to that seen with PI3-K inhibition is sufficient to induce blastocyst apoptosis and pregnancy failure we examined the effect of iodoacetate, an inhibitor of the glycolytic enzyme GAPDH, on embryo survival and pregnancy outcome. Previously the inhibition of GAPDH was shown to result in the accumulation of glycolytic intermediates upstream of the conversion of glyceraldehyde 3-phosphate to 1,3 bisphosphoglycerate by GAPDH (55,56). In addition the inhibition of GAPDH has been shown to lead to increased flux through the polyol and hexosamine pathways and has been suggested to lead to diabetic embryopathy (57,58). Wentzel et al. 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 (58). We demonstrated that the inhibition of GAPDH in blastocysts and TS cells resulted in decreased cell survival. In addition blastocysts cultured in iodoacetate displayed greater rates of fetal resorptions as compared to controls. Thus the inhibition of glycolysis alone is sufficient to explain the physiologic outcomes of inhibiting the PI3-K pathway in preimplantation embryos. We conclude that one mechanism by which PI3-K promotes cell survival and reproductive success during early development is through the maintenance of glucose metabolism.

Herein we demonstrate that the PI3-K pathway plays a critical role in embryo survival. In addition these data establish that PI3-K regulates cellular glucose metabolism 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. Further analysis is underway to determine whether this key survival pathway is inhibited during the preimplantation period in 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

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   IUBMB Life 51(5), 309-314

FIGURE LEGENDS

Fig. 1. Inhibition of PI3-K results in the induction of apoptosis in blastocysts. 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) Percentage of TUNEL-positive nuclei 
demonstrating DNA fragmentation per total embryonic nuclei. The 500 µM treatment group was 
determined to be statistically different (denoted by * p<0.001) from controls.

Fig. 2. Trophoblast stem (TS) cells treated with LY-294002 undergo apoptosis. TS cells were cultured in 
the presence of either DMSO (24-hour time point is depicted in the figure) or 150 µM LY-294002 for 0, 
6, 12 or 24 hours. The TUNEL assay was performed and the cells were analyzed using a BD 
FACSCalibur flow cytometer. An arbitrary gate was drawn and the percentage of cells within the gate 
was determined using Cell Quest software. The 12 and 24-hour LY-294002 treatments were found to be 
statistically different from the DMSO treatment group (denoted by *p<0.001, **p<0.0001).

Fig. 3. Inhibition of PI3-K results in decreased GLUT1 expression at the plasma membrane. A) 
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). B) TS cells were cultured in the presence of 
either DMSO or 150 µM LY-294002 for 12 hours and plasma membrane fractions were isolated. 2 µg of 
protein was loaded per lane on a 12% SDS-PAGE gel and analyzed by Western blot using an anti-GLUT1 
antibody. The membrane was simultaneously probed with an anti-actin antibody to normalize for protein 
loading.

Fig. 4. LY-294002 treatment results in decreased 2-deoxyglucose uptake in blastocysts. Blastocysts were 
cultured in the presence of DMSO or 250 µM LY-294002 for 30 hours. Individual blastocysts were then 
subjected to a 2-deoxyglucose uptake assay. 2-deoxyglucose uptake in the LY-294002 treated blastocysts 
was found to be statistically different from the control group (denoted by *, p<0.001).

Fig. 5. PI3-K inhibition leads to decreased hexokinase activity in blastocysts. For the hexokinase 
measurements, blastocysts were cultured in vitro in the presence of DMSO or 250 µM LY-294002 for 30 
hours and hexokinase levels were measured on individual blastocysts. Hexokinase activity in the LY-
294002 treated blastocysts was found to be statistically different from the control group (denoted by *, 
p<0.001).

Fig. 6. Inhibition of the PI3-K pathway at the blastocysts stage results in an increased frequency of fetal 
resorptions. A) Blastocysts were recovered and then cultured in vitro for 24 hours 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. 

B) The number of resorptions per number of implantation sites was quantitated and the resulting percentage of fetal resorptions is depicted in Panel B. The LY-294002 treated blastocysts were found to be statistically different from the control group (denoted by *, **, p<0.0001).

**Fig. 7.** Inhibition of GAPDH results in the induction of apoptosis in blastocysts. 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) Percentage of TUNEL-positive nuclei demonstrating DNA fragmentation per total embryonic nuclei. The 7 µM treatment group was determined to be statistically different (denoted by * p<0.0001) from controls.

**Fig. 8.** Iodoacetate induces apoptosis in TS cells. TS cells were cultured in the presence of either HTF media alone (24-hour time point is depicted in the figure) or 20 µM iodoacetate for 6, 12 or 24 hours. The TUNEL assay was performed and the cells were analyzed using a BD FACSCalibur flow cytometer. An arbitrary gate was drawn and the percentage of cells within the gate was determined using Cell Quest software. The 6, 12 and 24-hour IA treatments were found to be statistically different from the control group (denoted by *p<0.002, **p<0.0001, ***p<0.0001).

**Fig. 9.** Inhibition of GAPDH at the blastocysts stage results in an increased frequency of fetal resorptions. A) Blastocysts were recovered and then cultured in vitro for 24 hours 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. B) The number of resorptions per number of implantation sites was quantitated and the resulting percentage of fetal resorptions is depicted in Panel B. The iodoacetate treated blastocysts were found to be statistically different from the control group (denoted by *, **, p<0.0001).

**FOOTNOTES**

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We would like to thank Mia Wallace and Edeltraut Plut (Washington University) for their expertise and support with the embryo transfer experiments. In addition we would like to thank Mary Bade for her photographic assistance.
Figure 2

A.

B.
Figure 3

A.

<table>
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<th>DMSO</th>
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B.

- GLUT-1: 75 kDa, 50 kDa, 37 kDa
- actin: ![Image](image7)
Figure 4

mmol/kg wet wt/15 minutes

DMSO  LY-294002

*
Figure 5

pmoles/embryo/hour

DMSO  LY-294002

*
Figure 6

A.

B.

DMSO

LY-294002

% Successful pregnancies

% Fetal resorptions

DMSO

LY-294002

Downloaded from http://www.jbc.org on September 1, 2017

* **
Figure 7

A. No Treatment  2.5 µM IA  5 µM IA  7 µM IA

B. % TUNEL positive nuclei per blastocyst

- No Treatment
- 2.5 µM IA
- 5 µM IA
- 7 µM IA

* denoted significance at p < 0.05
Figure 8

A.

B.
Figure 9

A. U-shaped structures

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<th>Control</th>
<th>Iodoacetate</th>
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B. Bar chart showing % Successful pregnancies and % Fetal resorptions

- Control
- Iodoacetate

* * *
PI3-kinase activity is critical for glucose metabolism and embryo survival in murine blastocysts
Joan K. Riley, Mary O. Carayannopoulos, Amanda H. Wyman, Maggie Chi and Kelle H. Moley

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