MOLECULAR MECHANISMS INVOLVED IN SERTOLI CELL
ADAPTATION TO GLUCOSE DEPRIVATION

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RUNNING HEAD:
SERTOLI CELL ADAPTATION TO GLUCOSE DEPRIVATION

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ABSTRACT

Sertoli cells provide the physical support and the necessary environment to germ cell development. Among the products secreted by Sertoli cells, lactate —the preferred energy substrate for spermatocytes and spermatids— is present. Considering the essential role of lactate on germ cell metabolism, it is supposed that Sertoli cells must ensure its production even in adverse conditions as those that would result from a decrease in glucose levels in the extracellular milieu. The aim of the present study was to investigate: a) a possible effect of glucose deprivation on glucose uptake and on the expression of glucose transporters in rat Sertoli cells and b) the participation of different signal transduction pathways in the above-mentioned regulation. Results obtained show that decreasing glucose levels in Sertoli cell culture medium provokes: i) an increase in glucose uptake accompanied by only a slight decrease in lactate production, ii) an increase in GLUT1 and a decrease in GLUT3 expression and iii) an activation of AMPK-, PI3K/PKB- and p38MAPK-dependent pathways. Additionally, by using specific inhibitors of these pathways, a possible participation of AMPK- and p38MAPK-dependent pathways in the regulation of glucose uptake and GLUT1 expression is shown. These results suggest that Sertoli cells adapt to conditions of glucose deprivation in order to ensure an adequate lactate concentration in the microenvironment where germ cell development occurs.

KEYWORDS: Testis, signal transduction, GLUT, Lactate
INTRODUCTION

The Sertoli cell plays a central role in the development of a functional testis. At puberty, Sertoli cells provide physical support and metabolic assistance that are absolutely necessary for meiosis and spermiogenesis. Even though the precise composition of the extracellular environment where germ cells develop is incompletely known, there are no doubts that lactate is present in this milieu and that its secretion by Sertoli cells is highly regulated. Since lactate is the preferred energy substrate for spermatocytes and spermatids (13, 28), it is supposed that Sertoli cells must ensure its production even in adverse conditions as those that would result from a decrease in glucose levels in the Sertoli cell extracellular milieu.

Several biochemical steps are involved in lactate production and it has been shown that glucose transport across the cell membrane is an important event for the whole process. A family of structurally related glycoproteins, designated as GLUT, is responsible for mediating passive glucose transport. To date fourteen glucose transporter genes have been identified encoding for the proteins GLUT1–GLUT14 (24). These glucose transporter isoforms exhibit sequence similarities, but differ in their regulation and tissue distribution. Several studies show that glucose transport can be modulated by hormones (30). Additionally, studies with cultured mammalian cells support the concept that glucose itself may regulate its own transport and metabolism (16). The latter conclusion was mostly reached by evidence of increased rates of glucose uptake in response to glucose deprivation. In this context, the effect of glucose deprivation on the regulation of GLUT1 and GLUT4 isoform expression in insulin-sensitive cells has been extensively analyzed (15, 17, 43, 47, 48). Moreover, in a tissue highly dependent on glucose metabolism but not insulin-responsive like the brain, it has been shown that glucose deprivation increases the levels of expression of GLUT1 and GLUT3 isoforms (1, 31).
So far, expression of three glucose transporter isoforms, GLUT1, GLUT3 and GLUT8, has been observed in Sertoli cells (2, 11, 45). GLUT8 has not been localized at the plasma membrane, thus excluding its role in glucose transport from the extracellular milieu (32, 34). On the other hand, GLUT1 and GLUT3 are present at the plasma membrane and it may be assumed that these glucose transporters mediate glucose incorporation into Sertoli cells. FSH and certain growth factors and cytokines, hormones that promote an increment in glucose incorporation into Sertoli cells, differentially regulate GLUT1 and GLUT3 expression (11). Changes in glucose levels in the extracellular milieu, in addition to hormones, may also constitute a signal for Sertoli cells to up-regulate its glucose transporter system in order to ensure lactate production for germ cell development.

It has been shown that exposure of eukaryotic cells to metabolic stress, such as nutrient deprivation, triggers specific adaptative responses. The intracellular signaling molecules that participate in these responses probably involve AMP-activated protein kinase (AMPK). The latter is a metabolic-sensing protein that is activated by an increase in the AMP:ATP ratio. AMPK is activated by allosteric binding of AMP and by phosphorylation by one of the upstream AMPK kinases (18). Once activated, AMPK regulates ATP levels by suppressing biosynthetic pathways –such as fatty acid and cholesterol biosynthesis– as well as by activating ATP-generating catabolic pathways –such as fatty acid oxidation and glycolysis. Other intracellular signaling systems that may be involved in metabolic response to nutrient deprivation are the stress activated p38MAPK (3) and the survival pathway PI3K/PKB (46).

We have previously observed that AMPK is broadly distributed in testicular cells and that activation of AMPK by AICAR increases glucose uptake in Sertoli cells (10). In addition, we have previously demonstrated that in Sertoli cells the PI3K/PKB signaling
pathway participates in several responses to hormones, particularly in the regulation of glucose uptake into the cell (27, 35, 36). The aim of the present study was to investigate: a) the effect of a decrease in glucose concentration in the extracellular milieu on the regulation of glucose uptake and expression of glucose transporters and b) the participation of different signal transduction pathways in the regulation of glucose transport in conditions of glucose deprivation in Sertoli cells.

MATERIALS AND METHODS

Materials

Tissue culture media was purchased from GIBCO BRL (Life Technologies Ltd, Rockville, MD, USA). [2,6-3H]-2-deoxy-D-glucose (2-DOG) was purchased from NEN (Perkin Elmer Life and Analytical Sciences, Connecticut, USA). Antibodies for phosphorylated Acetyl-CoA Carboxylase (Ser79; P-ACC), phosphorylated protein kinase B (Ser473; P-PKB) and phosphorylated p38MAPK (Thr180/Tyr182; P-p38MAPK), total ACC, total PKB and total p38MAPK were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Compound C, SB203580 and wortmannin were purchased from Biomol (Plymouth Meeting, PA, USA). Kodak X-Omat S films were purchased from Eastman Kodak (Rochester, NY, USA). All other drugs and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sertoli cell isolation and culture

Twenty-day-old Sprague-Dawley rats were used to prepare Sertoli cells. Animals were housed and used according to the guide-lines recommended by the National
Institute of Health and approved by the Institutional Ethical Committee. Animals were killed by asphyxiation with CO\textsubscript{2} and decapitated. Testes were removed, decapsulated and Sertoli cell isolation was performed as previously described (26). Briefly, decapsulated testes were digested with 0.1\% collagenase and 0.006\% soybean trypsin inhibitor in Hanks’ balanced salt solution for 5 minutes at room temperature. Seminiferous tubules were saved, cut and submitted to 1 M glycine-2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase for 10 minutes at room temperature to remove germinal cells. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium which consists in a 1:1 mixture of Ham’s F12 and Dulbecco’s Modified Eagle medium, supplemented with 20 mM HEPES, 100 IU/ml penicillin, 2.5 \mu g/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 \mu g/ml transferrin, 5 \mu g/ml insulin, 5 \mu g/ml vitamin E and 4 ng/ml hydrocortisone. Sertoli cells were cultured in 6-, 24- or 96-multiwell plates or in 25 cm\textsuperscript{2} tissue culture flasks (5 \mu gDNA/cm\textsuperscript{2}) at 34°C in a mixture of 5\% CO\textsubscript{2}: 95\% air.

No myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to Sertoli cell cultures using a specific antiserum to alpha smooth muscle actin. Remaining cell contaminants were of germ cell origin and this contamination was below 5\% after 48 hours in culture as examined by phase contrast microscopy.

**Culture conditions**

Sertoli cells were allowed to attach for 48 hours in the presence of insulin and medium was replaced at this time with RPMI glucose-free medium with the addition of 100 IU/ml penicillin, 2.5 \mu g/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 \mu g/ml transferrin, 5 \mu g/ml vitamin E and 4 ng/ml hydrocortisone and supplemented with
variable concentrations of glucose. Cells harvested on day 5 were utilized for RNA isolation and the 48-hour conditioned media obtained at that time were used to evaluate lactate production. For 2-deoxyglucose uptake studies, cell monolayers on day 5 of culture, previously treated for variable periods of time (48, 24 and 6 hours) with different concentrations of glucose ranging from 0 to 8 mM glucose were used. Cells harvested on day 5 previously treated as indicated in figure legends were used to obtain cell extracts for Western blot analysis.

Measurement of 2-deoxy-D-glucose uptake

Glucose transport was studied using the uptake of the labeled nonmetabolizable glucose analogue 2-deoxy-D-glucose (2-DOG). Cells were washed three times with glucose-free PBS at room temperature. Then, Sertoli cells were incubated at 34°C in 0.5 ml glucose-free PBS containing [2,6-\(^3\)H]-2-DOG (0.5 μCi/ml) for 30 min. Unspecific uptake was determined in incubations performed in the presence of a 10000-fold higher concentration of unlabeled 2-DOG. At the end of the incubation period, dishes were placed on ice and extensively washed with ice-cold PBS until no radioactivity was present in the washings. Cells were then dissolved with 0.5 N sodium hydroxide, 0.4% sodium deoxycholate and the radioactivity was measured in a liquid scintillation counter (1214-RackBeta, Wallac). Parallel cultures receiving identical treatments to those performed before the glucose uptake assay were destined to DNA determinations. Results were expressed on a per μg DNA basis.

Lactate determination
Lactate was measured by a standard method involving conversion of NAD to NADH determined as the rate of increase of absorbance at 340 nm. A commercial kit from Sigma-Aldrich (St Louis, MO, USA) was used.

**Northern blot analysis**

Northern blot analysis was carried out in total RNA samples isolated from Sertoli cells cultured in 25 cm² tissue culture flasks by the guanidinium isothiocyanate method (4). The amount of RNA was estimated by spectrophotometry at 260 nm. About 15 μg total RNA was electrophoresed on a 1% agarose-10% formaldehyde gel. After migration, RNAs were transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech, Buenos Aires, Argentina) by capillary transfer with 20 x SSC (20 x stock solution: 3 M NaCl and 0.3 M sodium citrate, pH 7.4) and fixed with U.V. Stratalinker (Stratagene Cloning Systems, La Jolla, CA, USA). cDNA probes (rat GLUT1 2.6 kb insert, EcoRI; mouse GLUT3 0.6 kb insert, EcoRI-HindIII; and 18S oligonucleotide) were labeled with [α-³²P]deoxy-CTP (NEN, Perkin Elmer Life and Analytical Sciences, Connecticut, USA) using a random-primed labeling kit (Prime-a-Gene Labeling System, Promega Corporation, Madison, USA). Blots were prehybridized for 5 h at 42°C in 50% formamide, NaCl/Pi/EDTA (0.75 M NaCl, 20 mM sodium phosphate (pH 7.5) and 1 mM EDTA), 5 x Denhart’s solution, 10% dextran sulfate, 0.5% SDS and 100 μg/ml herring sperm DNA. Hybridization was then performed overnight at 42°C in the same hybridization buffer containing 1-4 x 10⁶ c.p.m./ml ³²P-labeled probe. Membranes were washed utilizing different astringency conditions depending on the probe utilized. Membranes were exposed to Kodak X-Omat S films M for 7 days at –70°C. The intensities of the autoradiographic bands were
estimated by densitometric scanning using NIH Image software (Scion Corporation, Frederick, MD). The 18S signal was used to standardize mRNA contents.

Cell extracts and Western blot analysis

Sertoli cells were washed once with PBS at room temperature. Then, 200 μl PBS containing 2 μl of a protease inhibitor cocktail from Sigma-Aldrich (P-8340), 1mM NaF, 1mM EGTA, 1mM EDTA and 2mM PMSF were added to the cells. Cells were then placed on ice and disrupted by ultrasonic irradiation. A 200 μl volume of 2X Laemmli buffer (4% w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, 0.004% w/v bromophenol blue and 0.125 M Tris-HCl, pH 6.8) was added and thoroughly mixed (20). Samples were immersed in a boiling water bath for 5 minutes and then immediately settled on ice. Proteins were resolved in 10% SDS-PAGE (10% acrylamide/bisacrylamide for the resolving gel and 4.3% acrylamide/bisacrylamide for the stacking gel) in a Mini Protean 3 Cell (Bio-Rad, Hercules, CA, USA). After SDS-PAGE, gels were equilibrated in transfer buffer for 10 minutes and electrotransferred at 100 V for 60 minutes onto PVDF membranes (Hybond-P, Amersham Pharmacia Biotech, UK) using a Mini Trans-Blot Cell (Bio-Rad). Membranes were probed with antibodies that recognize phosphorylated ACC (Ser79) – a downstream target of AMPK –, phosphorylated PKB (Ser473) and phosphorylated p38-MAPK (Thr180/Tyr182) or with antibodies that recognize total ACC, PKB and p38-MAPK. A 1:1000 dilution of primary antibodies was used. For chemiluminescent detection of the blots, a commercial kit from Cell Signaling Technology was used. The intensities of the autoradiographic bands were estimated by densitometric scanning using NIH Image software. As loading controls in the electrophoresis total ACC, PKB and p38MAPK levels were used.
Other assays

DNA was determined by the method of Labarca & Paigen (19).

Statistical analysis

To analyze data from glucose uptake studies and from lactate production, one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons were performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). Probabilities <0.05 were considered statistically significant.

RESULTS

Effect of decreasing glucose levels in the extracellular milieu on glucose uptake and lactate production in rat Sertoli cells.

In order to analyze the effect of glucose levels in the extracellular milieu on glucose uptake and on lactate production, Sertoli cells were cultured for 48 h in media containing different concentrations of glucose ranging from 8 mM (the glucose concentration currently used in the culture media of Sertoli cells) to 0 mM. These culture conditions did not modify Sertoli cell viability as evaluated by trypan blue exclusion (data not shown).

Figure 1A shows that an increase in glucose uptake at concentrations of glucose equal to or lower than 4 mM in the culture medium was observed. To analyze the kinetics of this effect of glucose deprivation on Sertoli cell glucose uptake, cells were incubated in glucose free media for 6, 24 or 48 h. Figure 1B shows that in the complete
absence of glucose in the cultured medium, cells increased its capacity to incorporate 2-
DOG in a time-dependent manner. A significant increase in glucose incorporation into
the cell was observed as soon as 6 h after glucose deprivation.

Figure 2 shows that a reduction in glucose concentration in the culture medium
produced a slight but significant decrease in lactate production and yet the production of
this glucose metabolite was observed even under the complete absence of glucose in the
culture medium.

**Effect of decreasing glucose concentrations on GLUT1 and GLUT3 mRNA levels
in rat Sertoli cells.**

The next set of experiments was designed to analyze the possible effect of
decreasing glucose levels in the culture medium on expression of the glucose
transporters GLUT1 and GLUT3. Cells were cultured for 48 h in media containing
variable concentrations of glucose. Figures 3A and 3B show respectively that a
diminution of glucose offer to the cell resulted in increased GLUT1 and decreased
GLUT3 mRNA levels.

Increased levels of mRNA may reflect increased transcription and/or
stabilization of pre-existing mRNA molecules. To determine if stabilization of mRNA
was a mechanism involved in the increase in GLUT1 mRNA levels observed under
glucose deprivation, cells were cultured in media containing 8 mM and 0 mM glucose
for 48 h and thereafter, incubated in the presence of actinomycin D for different periods
of time. Figure 4 shows that in conditions of glucose deprivation, GLUT1 mRNA
stability increased.
Effect of glucose deprivation on the regulation of signal transduction pathways in Sertoli cells.

We next analyzed possible signal transduction pathways that could potentially be activated in response to glucose deprivation. Three signaling pathways –AMPK, p38MAPK and PI3K/PKB– related respectively to energetic metabolism, cell stress and cell survival were evaluated. Sertoli cells were cultured in parallel with 8 mM glucose and with glucose free media for variable periods of time (15, 30 and 60 minutes). The levels of phosphorylated Acetyl-CoA Carboxylase (P-ACC), a downstream substrate of activated AMPK, phosphorylated p38MAPK (P-p38MAPK) and phosphorylated protein kinase B (P-PKB) were analyzed by Western blot. Figure 5 shows that glucose deprivation produced a time-dependent increment in P-ACC, P-p38MAPK and P-PKB levels. Pooled data obtained in three independent experiments performed in 15 minute incubations without glucose revealed 2.3±0.2 and 5.3±0.9 fold-stimulation (mean±SD) in P-ACC and P-PKB respectively. Additionally, a 2.2±0.5 fold-stimulation in P-p38MAPK levels in 30 minute incubations was observed.

Participation of AMPK, p38MAPK and PI3K/PKB in the regulation of glucose uptake and GLUT1 mRNA levels induced by glucose deprivation.

We next examined whether those signaling pathways which became activated under conditions of glucose deprivation participated in the regulation of glucose uptake and GLUT1 mRNA levels. Specific signal transduction pathway inhibitors, Compound C, SB203580 and wortmannin, were used to evaluate the participation of AMPK, p38MAPK and PI3K/PKB respectively. Wortmannin did not modify 2-DOG glucose uptake in response to glucose deprivation (Table 1). Figures 6A and 6B show that Compound C and SB203580 decreased 2-DOG uptake in response to glucose
deprivation. These figures also show that in the presence of 8 mM glucose in the culture medium the inhibitors did not modify 2-DOG uptake. Finally, Figure 6C shows that Compound C and SB203580 decreased the levels of GLUT1 mRNA in conditions of glucose deprivation.

DISCUSSION

The process of spermatogenesis and consequently male fertility is dependent upon testicular somatic cells. Leydig cells are essential for androgen production whereas Sertoli cells, the main component of the so called blood-testis barrier, are essential for the provision of an adequate and protected environment for germ cell development. The majority of the germ cells is situated beyond the blood testis barrier and relies on Sertoli cell production of factors to fuel their metabolism (49). In this context, lactate produced by Sertoli cells is delivered to and used by germ cells as an energy substrate (13, 28, 37).

As mentioned in the introduction, we hypothesized that Sertoli cells would ensure lactate production even in adverse conditions, as those resulting from a decrease in glucose levels in the extracellular milieu. Glucose is not the main energy substrate for Sertoli cells (50) and this fact may explain why Sertoli cells maintain viability in culture in the complete absence of glucose. The hypothesis that Sertoli cells would ensure lactate offer to germ cells in these adverse conditions was confirmed by the fact that Sertoli cell lactate production was still observed when glucose levels were low and even in the complete absence of glucose in the culture medium. The production of lactate in the absence of glucose in the extracellular milieu, a result that is seemingly surprising, may be the consequence of metabolism of glucose originated from certain aminoacids or alternatively by glycogen metabolism. Noteworthy, and making the possibility that glycogen hydrolysis
fuels the glycolytic pathway feasible, histochemical evidence of the presence of glycogen and of glycogen phosphorylase activity in Sertoli cells have been demonstrated (22, 41).

It has been observed that glucose deprivation leads to a compensatory increase in the glucose transport system in order to optimize glucose utilization and to maintain energy levels in some cell types (16). Accordingly, we have observed that treatment of Sertoli cells with decreasing levels of glucose in the extracellular milieu was always accompanied by an augmentation in the capacity to incorporate 2-DOG into the cells. The increase in glucose uptake is due to increased levels of active glucose transporters at the plasma membrane. This increase in GLUTs may occur by translocation or activation of pre-existing transporters and/or by increased expression of these molecules. No attempts were made to study translocation or activation of preexisting transporters but we did study transporter expression. In other tissues, augmented GLUT1 mRNA levels under glucose deprivation conditions have been observed (17, 40, 43, 48, 51). The working knowledge of the regulation of GLUT4 in insulin-sensitive cells is somehow controversial (15, 17, 43) and few data are available on the regulation of other glucose transporters in response to glucose deprivation (7, 31). We have observed an increase in GLUT1 and a decrease in GLUT3 expression in Sertoli cells as a consequence of withdrawing glucose from the culture medium. While increased GLUT1 expression has been previously observed under similar experimental conditions in other cell types, the negative regulation of GLUT3 has not been previously reported. Considering that we observed activation of AMPK under conditions of glucose deprivation and that we have previously demonstrated that pharmacological activation of AMPK provokes a decrease in GLUT3 expression (10), it is tempting to speculate that down-regulation of GLUT3 is the result of AMPK activation under these experimental conditions.
Regulation of mRNA stability plays a major role in the control of GLUT1 gene expression (33). RNA-binding proteins that recognize specific consensus sequences are thought to play a role in the stabilization process. It has been observed that up-regulation of RNA-binding proteins with apparent binding specificity for the GLUT1 3’UTR is involved in the stabilization of the mRNA (25, 44). Alteration in the stability of the GLUT1 transcript in conditions that include inhibition of oxidative phosphorylation, glucose deprivation and hormone stimulation has been previously observed in other cell types (1, 5, 23, 39). In Sertoli cells, the experiments performed in the presence of a transcription inhibitor showed a lower decay of GLUT1 mRNA in conditions of glucose deprivation, suggesting that, also in this cell type, the increment in GLUT1 mRNA levels can be partly explained by mRNA stabilization.

An intriguing question is which intracellular mechanisms are triggered as a consequence of glucose deprivation in Sertoli cells. Various signaling pathways, such as ERK1/2, JNK, AMPK, PI3K/PKB and p38MAPK may result activated in response to glucose deprivation (3, 47, 51). In the present study, three signaling pathways that have been related to energetic metabolism, cell stress and cell survival were analyzed in Sertoli cells. Results presented herein indicate that AMPK, p38MAPK and PI3K/PKB become activated shortly after the removal of glucose in Sertoli cells. These protein kinases have been linked to the regulation of glucose uptake elicited by diverse hormonal stimuli in several cell types (6, 8, 14, 42).

The relationship between AMPK activation and GLUT1 expression has been documented in skeletal muscle cells transfected with a constitutively active mutant form of the catalytic α subunit of AMPK and in DU145 prostate carcinoma cells stimulated with AICAR (8, 51). As for the participation of p38MAPK in glucose uptake and GLUT1 expression, it has been reported that overexpression of a constitutively active MKK6,
which activates p38MAPK, increases glucose uptake and GLUT1 expression in 3T3-L1 adipocytes and L6 myotubes (9).

The present study shows that AMPK and p38MAPK activation may be related to the increase in glucose uptake and GLUT1 expression induced by glucose deprivation in Sertoli cells. Evidence of the participation of these signaling pathways arose from experiments performed in the presence of specific inhibitors such as Compound C and SB203580. The results obtained on the participation of AMPK in glucose uptake are in agreement with previous observations showing that pharmacological activation of AMPK with AICAR results in an increase in glucose uptake which is accompanied by increased GLUT1 expression in Sertoli cells (10).

On the other hand, the PI3K/PKB signaling pathway, which is also activated under conditions of glucose deprivation, does not apparently participate in the regulation of glucose uptake under these experimental conditions. This conclusion arose from the observations made on the lack of effect of wortmannin—an inhibitor of PI3K—on glucose uptake. The latter results are somehow surprising considering that the PI3K/PKB pathway is widely used to regulate glucose uptake in response to several hormonal stimuli in Sertoli cells (27, 35, 36). On the other hand, results presented herein are in agreement with the observations made in muscle cells under conditions of hypoxia where stimulation of glucose uptake independently of PI3K and involving an AMPK-dependent pathway has been observed (12, 21, 29, 38).

Even though observations in Sertoli cell cultures may not exactly represent the in vivo situation, the results presented herein suggest that an adaptation of Sertoli cells exists under conditions of decreasing glucose supply. This adaptation would be finally destined to ensure adequate concentrations of lactate in the microenvironment where germ cell development occurs.
ACKNOWLEDGEMENTS

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GRANTS

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35. **Riera MF, Galardo MN, Pellizzari EH, Meroni SB, Cigorraga SB.** Participation of phosphatidyl inositol 3-kinase/protein kinase B and ERK1/2 pathways in interleukin-


FIGURE LEGENDS

Figure 1: Effect of decreasing glucose levels on glucose uptake in rat Sertoli cells.
A) Sertoli cells were cultured for 48 hours in medium contained variable concentrations of glucose (8, 4, 2 or 0 mM). B) Sertoli cells were incubated for variable periods of time (6, 24 and 48 hours) in medium containing 8 mM or 0 mM glucose. Glucose uptake assay (2-DOG uptake) was performed after these incubations periods. The results represent the mean ± SD of triplicate incubations in one representative experiment out of three (* p< 0.05 vs 8 mM glucose).

Figure 2: Effect of decreasing glucose levels on lactate production in rat Sertoli cells. Sertoli cell were cultured for 48 hours in medium containing variable concentrations of glucose (8, 4, 2 or 0 mM). Lactate was determined in the 48-hour conditioned media. Results are expressed as mean ± SD of triplicate incubations in one representative experiment out of three. Different letters indicate statistically significant differences (p< 0.05).

Figure 3: Effect of decreasing glucose levels on GLUT1 and GLUT3 mRNA levels in rat Sertoli cells. Sertoli cells were incubated for 48 hours in medium containing variable concentrations of glucose (8, 4, or 0 mM). Total cellular RNAs were extracted and Northern blot analysis was performed. Membranes were hybridized with labeled cDNA probes for GLUT1 (A) and GLUT3 (B). The upper panels show a representative experiment out of three. The lower panels show pooled data of three independent experiments indicating the fold-variation in GLUT1 and GLUT3 mRNA levels found in
cultures performed with 4 and 0 mM glucose when compared with cultures performed with 8 mM glucose. Results are expressed as mean ±SD.

**Figure 4: Effect of glucose deprivation on GLUT1 mRNA stability.** Sertoli cells were incubated for 48 hours in medium contained 8 or 0 mM glucose. Then, actinomycin D (Act D, 5μg/ml) was added and cells were further incubated for additional 1, 3 or 5 hours. Total cellular RNAs were extracted at the time of Act D addition and at the indicated times described above and Northern blot analysis was performed. Membranes were hybridized with labeled cDNA probes for GLUT1. The upper panels show a representative experiment out of two. The lower panel shows GLUT1 mRNA levels remaining 1, 3 and 5 hours after Act D addition in cultures performed in 8 and 0 mM glucose.

**Figure 5: Effect of glucose deprivation on the levels of phosphorylated ACC, p38MAPK and PKB.** Sertoli cells were cultured in medium containing 8 mM glucose or 0 mM glucose for variable periods of time (15, 30 and 60 min). Cell extracts were utilized for Western blot analysis using antibodies specific for: A) phosphorylated ACC (P-ACC) and total ACC (T-ACC), B) phosphorylated p38MAPK (P-p38) and total p38MAPK (T-p38) and C) phosphorylated PKB (P-PKB) and total PKB (T-PKB). Autoradiographies show a representative experiment out of three. Bar graphs show pooled data of three independent experiments indicating fold-variation in phosphorylation (mean ±SD).

**Figure 6: Effect of AMPK and p38MAPK inhibitors on glucose deprivation-stimulated glucose uptake and GLUT1 mRNA levels.** Sertoli cells were incubated for
48 hours in medium containing 8 mM or 0 mM glucose: A) in the absence or presence of Compound C (25 and 50 μM) and B) in the absence or presence of SB203580 (1 and 10 μM). Results are expressed as mean±SD of triplicate incubations in one representative experiment out of three. Different letters indicate statistically significant differences in glucose uptake (p<0.05). The lower autoradiograph (C) shows GLUT1 expression in Sertoli cells incubated for 48 hours in medium contained 8 mM or 0 mM glucose in the absence or presence of Compound C (50 μM) or SB203580 (10 μM). Numbers at the bottom of each lane indicate fold-variation in GLUT1 mRNA levels relative to 8 mM glucose. This experiment was repeated twice.
**A**

*Glucose (mM)*

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<th>8</th>
<th>4</th>
<th>2</th>
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<td>1200</td>
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*B*

*8mM Glucose*  
*0mM Glucose*

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<td>800</td>
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* (statistically significant)
Glucose (mM)
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<td>18S-</td>
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GLUT1/18S (fold variation):

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GLUT3/18S (fold variation):

B
GLUT1- 18S-

8 mM Glucose

18S-

0 mM Glucose

Act D (h)

% GLUT1 remaining

0 1 3 5

Act D (h)

8 mM Glucose

0 mM Glucose
A

GLUCOSE UPTAKE (dpm/gDNA)

Comp C (μM)

B

GLUCOSE UPTAKE (dpm/gDNA)

SB203580 (μM)

C

0mM

8mM +CC +SB

GLUT1-

18S-

1.0 1.8 0.8 1.1
Table 1. Effect of a PI3K inhibitor on glucose deprivation-stimulated glucose uptake.

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<tr>
<td>8 mM + W 0.01μM</td>
<td>892±71(^a)</td>
</tr>
<tr>
<td>8 mM + W 0.1μM</td>
<td>846±65(^a)</td>
</tr>
<tr>
<td>0 mM</td>
<td>1513±103(^b)</td>
</tr>
<tr>
<td>0 mM + W 0.01μM</td>
<td>1535±98(^b)</td>
</tr>
<tr>
<td>0 mM + W 0.1μM</td>
<td>1575±101(^b)</td>
</tr>
</tbody>
</table>

Sertoli cells were incubated for 48 hours in medium contained 8 mM or 0 mM glucose in the absence or presence of two different wortmannin concentrations (W, 0.01 and 0.1μM). 2-DOG uptake assay was performed after the 48 hour incubation period. Results are expressed as mean±SD of triplicate incubations in one representative experiment out of three. Different letters indicate statistically significant differences (p<0.05).