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Original Research

Glucose restriction reverses the Warburg effect and modulates PKM2 and mTOR expression in breast cancer cell lines

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Abstract: Aerobic glycolysis, known as the "Warburg effect", is one of several hallmarks of cancer cells. The conversion of phosphoenolpyruvate (PEP) to pyruvate can be down regulated by the re-expression of the embryonic isoform 2 of pyruvate kinase (PKM2). This mechanism allows the accumulation of glycolytic intermediates for the biosynthesis of macromolecules, such as proteins, lipids and nucleic acids. PKM2 is favored by the well-known PI3K/Akt/mTOR proliferative pathway. This pathway is induced by high glucose levels, and the mTOR kinase is the central activator of the Warburg effect. In this study, we investigated the role of glucose restriction (GR) and mTOR inhibition in reversing the Warburg effect in MDA-MB 231 and MCF-7 breast cancer cell lines. PKM2 expression was measured by western blot. Lactate production by cells was determined by a colorimetric assay. The concentration of glucose in the supernatant of cells was measured using the Trinder method. ATP level was evaluated by using a Colorimetric/Fluorometric ATP Assay Kit. Our results showed that MDA-MB 231 cells increased glucose consumption when the glucose concentration was 0 g/L ($P < 0.01$). In MCF-7 cells, glucose deprivation reduced lactate secretion by 80% ($P = 0.0001$) but tripled glucose consumption ($P = 0.0041$). ATP concentration increased approximately when MCF-7 cells were deprived of glucose ($P = 0.02$). GSK1059615 does not significantly modulate lactate secretion and glucose uptake in both cell lines. Glucose restriction contribute to the reduction of the Warburg effect through mTOR inhibition and regulation of PKM2 kinases.

Key words: Glucose restriction; PKM2; mTOR; MDA-MB 231; MCF-7; GSK1059615.

Introduction

Cellular metabolism is a fundamental subject of research. For many years, researchers have focused on understanding how cancer cells meet their metabolic and energetic needs. Metabolic regulation distinguishes normal cells from cancer cells. Most differentiated cells convert glucose to pyruvate via glycolysis, and pyruvate then enters the mitochondria where it is transformed into acetyl-CoA by the pyruvate dehydrogenase (PDH) enzyme complex. Through the Krebs cycle, acetyl-CoA powers the production of energy in the form of ATP through oxidative phosphorylation in the respiratory chain of mitochondria. Tumor cells, however, have a different metabolic profile. Metabolic changes occurring within cancer cells were first described in 1927 by Otto Warburg (1). Warburg's work showed that, unlike normal cells, tumor cells convert glucose into lactate through the glycolytic pathway, even in the presence of sufficient oxygen (aerobic glycolysis). The metabolic "shift" from oxidative phosphorylation to aerobic glycolysis, now known as the Warburg effect, has been confirmed in many cancers. While glycolysis provides only 2 molecules of ATP, it effectively provides metabolic intermediates and precursors required to promote the metabolism of cancer cells (2). Because glycolysis is 18 times less efficient than oxidative phosphorylation in producing ATP, cancer cells must increase glucose uptake and oxidation. PET (positron emission tomography), a diagnostic and clinical tool, has demonstrated increased glucose consumption in tumor cells (3). To explain the metabolic changes in cancer cells, Warburg hypothesized that mitochondrial dysfunction in cancer cells forces them to turn to glycolysis for survival (4). In 2010, Cairns suggested that if metabolic changes in cancer cells mainly functioned to provide for the proliferative needs of the cells, then three key aspects would be required: rapid obtaining of ATP, biosynthesis of macromolecules and maintenance of redox status compatible with cell survival (5).

Unlike normal cells, cancer cells do not change their metabolism in response to growth factors. They accumulate genetic changes associated with the activation of proto-oncogenes or inactivation of tumor suppressor genes, thereby becoming independent of growth factors. These genetic changes have long been recognized as capable of modifying cell proliferation and survival. However, it has been shown that most of these modifications also play a metabolic role in promoting proliferation.

Although metabolism in cancer cells may vary according to these genetic abnormalities and by cancer

type, some proteins appear to be essential for tumor growth and are thus universally expressed.

Pyruvate kinase (PK) is the last enzyme in glycolysis and catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate; this is accompanied by ATP production. PK is expressed as four different isoforms: type M1 (PKM1), type M2 (PKM2), type L (PKL, liver) and type R (PKR, red blood cell). PKM1 and PKM2 represent two splice variants of the PKM gene. Type L and type R isoforms represent two splice variants of the PKL gene and are regulated by different tissue-specific promoters. Pyruvate kinase isoform R is expressed in erythrocytes, while pyruvate kinase isoform L is expressed in tissues with high gluconeogenesis activity, such as liver and kidney (6). PKM1 is expressed in tissues with high energy demand, such as skeletal muscle and brain. It has the highest affinity for its substrate PEP and is not allosterically regulated or phosphorylated (7).

PKM2 is expressed in tissues with a high rate of nucleic acid synthesis, such as fetal embryonic and adult stem cells tissues (8). It was discovered in a hepatoma cell line, and elevated expression of PKM2 has been observed in numerous cancerous cells (9). PKM2 activity and substrate affinity for PEP depends on its quaternary structure, which is governed by several intermediate metabolites (7). Studies on the mechanisms for expressing PKM2 in cancer cells are limited. However in 2010, David reported that the Myc oncogene may preferentially activate the expression of PKM2 (10). Thus, Myc increases the expression of hnRNPs (heterogeneous nuclear ribonucleoproteins), which were found to bind to intronic sequences flanking exon 9 and resulted in exon 10 inclusion in Hela cells (10). The production of PKM2 instead of PKM1 seems paradoxical because the enzymatic activity of PKM2 is much lower than that of PKM1, suggesting lower ATP production. However, the expression of PKM2 could favor anabolic pathways and slow glycolysis, thus allowing the accumulation of metabolites and channeling them towards other pathways such as the pentose phosphate pathway (PPP) and serine synthesis (11). Recently, the role of PKM2 has been shown not to be limited to cellular metabolism. Indeed, PKM2 could also play a role at the nuclear level through promoting the activation of hypoxia inducible factor (HIF-1α) and its target genes, especially those activating aerobic glycolysis (12).

A key upstream activator of the inactive PKM2 isoform is phosphoinositide 3-kinase (PI3K), the enzyme responsible for generating 3-phosphorylated phosphoinositides and activating protein kinase B (Akt) (13). Akt activation is a biological indicator of the aggressiveness of cancer cells (14), and preclinical studies have shown that generic inhibitors of PI3K, such as wortmannin and LY294002, induce apoptosis and inhibit tumor growth (15). PI3K signaling also involves mammalian target of rapamycin (mTOR), a downstream target of Akt. Activation of mTOR has been observed in most cancers (16), and activation of this pathway causes major metabolic changes at several levels. Regarding glycolysis, activation of the PI3K/Akt/mTOR pathway stimulates the expression of HIF-1α and c-Myc (17) and glucose transporters (such as GLUT1 and GLUT4) (18).

Tumor formation seems to involve alterations in the expression of PKM isoforms, changes in PK activity and a metabolic switch from oxidative phosphorylation to aerobic glycolysis.

In this report, we investigated the role of glucose restriction om Mtor and PKM2 modulation in two breast cancer cell lines, the MDA-MB-231 and the MCF-7. The energy production has been also monitored through the ATP intracellular ATP concentration.

Materials and Methods

Cell culture and glucose restriction

Established human triple-negative breast carcinoma cells (MDA-MB 231), and human-estrogen receptor-positive breast carcinoma cells (MCF-7) were purchased from

American Type Culture Collection (ATCC). All MDA-MB 231 and MCF-7 cells were cultured in DMEM (Sigma-Aldrich) with different glucose concentrations (0, 1 and 4.5 g/l) and supplemented with 10% FBS (fetal bovine serum, Sigma-Aldrich, Taufkirchen, Germany), 1% Pen-Strep (penicillin-streptomycin, Sigma-Aldrich, Taufkirchen, Germany) and 2 mM glutamine (Sigma-Aldrich, Taufkirchen, Germany). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells were pre-incubated for five days in the appropriate medium, and treatment occurred when cells reached 80% confluence.

Western blot analysis

Protein lysates from breast cancer cell lines were prepared in lysis buffer (150 mM NaCl, 2 mM EDTA, 0.5% deoxycholate sodium, 0.1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH = 8) supplemented with protease and phosphatase inhibitors. Proteins were separated on Tris-Glycine gradient polyacrylamide gels (Abcam) and transferred onto Immuno-Blot PVDF membranes. Membranes were incubated in blocking buffer (1X TBS containing 5% milk and 0.05% Tween-20) for 1 hour, probed with antibodies specific for PKM2 (Abcam, 1:5000 dilution), or β-actin (Santa Cruz, 1:1000 dilution), washed, and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). Antibody binding was detected by incubation with enhanced chemiluminescence (ECL) reagents (*Abcam*) and exposure of the membrane in an ECL machine. The expression of the desired protein was compared to that of β-actin, which served as an internal control.

Briefly, MDA-MB 231 and MCF-7 cells incubated with different amounts of glucose were treated with 20 nM GSK1059615 for 48 h. After treatment, the cells were harvested in PBS and lysed for 15 min at 4°C in the aforementioned lysis buffer. The supernatant containing the protein was collected, and the protein concentration was measured using the Bradford assay. To evaluate the expression of PKM2 (58 KDa), 40 µg of proteins were separated in 10% SDS-PAGE gel

Flow cytometry analysis of mTOR protein levels

To determine the intracellular protein levels mTOR following GSK1059615 treatment in 0, 1 and 4.5g/L of glucose, cells were washed twice with PBS, then fixed and permeabilized using the Cytofix/ Cytoperm™Kit (BD, USA) according to the manufacturer's instruc-

tions. Briefly, cells were incubated with a fixation permeabilization solution for 20 minutes at 4˚C. After incubation, cells were washed twice with Perm/Wash™ buffer, and then stained with the monoclonal antibody against total mTOR (Cell Signaling), for 30 minutes at 4˚C in the dark. Finally, the cells were washed twice with Perm/Wash™buffer and re-suspended in ranging buffer for the flow cytometry analysis.

Colorimetric determination of L-lactate

Lactate production by cells was colorimetrically measured in normoxia using a Lactate-PAP Kit (BioMériaux). Lactate PAP detects L-lactate using the lactate oxidase-peroxidase-chromogen sequence and the subsequent conversion of hydrogen peroxide to quinoneimine, a colored product:

$$\text{L-lactate} + O_2 \xrightarrow{\text{Lactate oxidase}} \text{pyruvate} + H_2O_2$$

$$H_2O_2 + \text{4-amino-antipyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + \text{4-chlorophenol} + HCl + 2H_2O$$

The intensity of color (due to quinoneimine) is measured at 505 nm and is proportional to the quantity of L-lactate present in the sample.

Briefly, MDA-MB 231 and MCF-7 cells were preincubated for five days at different glucose concentrations and seeded in a six-well plate at a density of 200,000 cells/well. After adherence, cells were treated with 20 nM GSK1059615 for 48 h. After treatment, lactate production was measured using 10 µL of the supernatant of each sample according to the manufacturer's instructions.

Measurement of glucose concentration

The concentration of glucose in the supernatant of treated cells was measured in the Saint Joseph University Laboratory of Biochemistry using the Trinder method (Biolabo). In this method, glucose is oxidized by glucose oxidase to gluconic acid and H_2O_2. The hydrogen peroxide generated then reacts with 4-chlorophenol and PAP (4-Amino-antipirine) in the presence of peroxidase to form a red quinoneimine. The absorbance of this colored complex at 500 nm is proportional to the glucose concentration in the culture medium.

Briefly, MDA-MB 231 and MCF-7 cells were preincubated for five days in medium containing different glucose concentrations and seeded in a six-well plate at a density of 200,000 cells/well. After adherence, cells were treated with 20 nM GSK1059615 for 48 h. After treatment, the glucose concentration in the culture medium was determined from 10 µL of supernatant from each sample. The concentration of glucose consumed was calculated by subtracting the concentration of glucose present in the culture medium before and after cell treatment.

Colorimetric assay of intracellular ATP

ATP produced by cell mitochondria was measured using a Colorimetric/Fluorometric ATP Assay Kit (Abcam). This simple detection method is based on the phosphorylation of glycerol to generate a colored product that is easily quantified by colorimetric assay. In short, MDA-MB 231 and MCF-7 cells were incubated at different glucose concentrations then treated with 20 nM GSK1059615 for 48 h. After treatment, 10^6 cells were solubilized with ATP Assay Buffer provided by the kit and centrifuged at 13,000 rpm for 2 minutes at 4°C. Proteins were removed from the supernatant in the presence of 4 M perchloric acid (PCA) and then centrifuged at 13,000 rpm for 2 minutes at 4°C. Approximately 50 µL of each sample were incubated with 50 µL of ATP Reaction Mix for 30 minutes at room temperature and away from light according to the manufacturer's instructions. The absorbance of the colored product was measured using a spectrophotometer at 570 nm.

Protein quantification

Protein concentration was measured using the Bradford method with a Bio-Rad Protein Assay Kit according to the manufacturer's recommendations. Briefly, serial dilutions of BSA at known concentrations were prepared as standards, and 5 mL of an acidic Coomassie Blue G-250 dye solution (5x dilution) were added to diluted proteins. The absorbance of the colored solution was measured with a spectrophotometer at 595 nm.

Statistical analysis

Statistical analysis was performed using t-tests with Graph-Pad Quickcalcs online statistical software (http://www.graphpad.com/quickcalcs/ttest1.cfm).

Results

Effect of glucose restriction (GR) and GSK1059615 treatment on the expression of PKM2

Because pyruvate kinase (PKM2) is an important mediator of the Warburg effect, we quantified PKM2 protein in both MDA-MB 231 and MCF-7 cell lines using Western blot analysis (Figure 1 A&B). Cells maintained in DMEM with various glucose concentrations were incubated with or without 20 nM GSK1059615 for 48 h. The data in figure 1C showed that reducing the glucose concentration in the medium to 0 g/L, de-

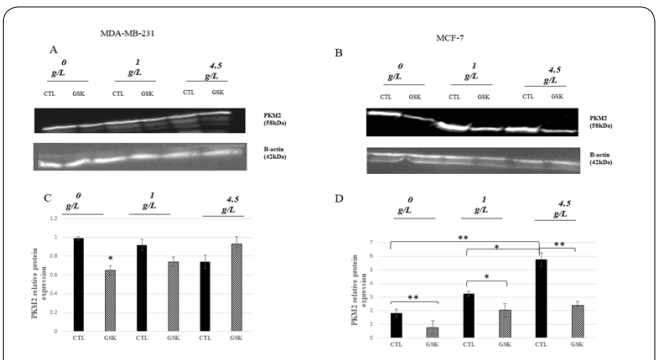

Figure 1. The effect of GR and GSK1059615 on the protein expression of PKM2. Cells were incubated in medium of varying glucose concentration in the presence or absence of 20 nM GSK1059615 for 48 h. After treatment, cells were lysed and 40 µg of extracted proteins were analyzed using Western blotting with β-actin (42 kDa) as an internal control for MCF-7 (Figure1A) and MDA-MB-231 (Figure 1B). Each value represents the average of three different experiments. Each value has its own control which was defined as 100%. Each point represents a mean + / _ SD for all experiments. *P< 0.05. ** P <0.01 compared to untreated cells. The relative protein expression was obtained by using the software Gelanalyzer (figures 1 C&D).

Table 1. The effect of GSK treatment on mTOR expression in different glucose concentrations (4.5g/L; 1g/L and 0g/L).

MCF-7	4.5g/L	CTL	11.81±16.19
		GSK	5.04±0.35
	1g/L	CTL	96.81±3.57
		GSK	77.10±28.23
	0g/L	CTL	39.01±8.44
		GSK	8.69±3.52
MDA-MB-231	4.5g/L	CTL	85.93±7.70
		GSK	83.21±5.03
	1g/L	CTL	89.21±3.06
		GSK	85.10±2.35
	0g/L	CTL	19.07±0.33
		GSK	4.86±4.40

MCF-7 and MDA-MB-231 cells were treated with 20 nM GSK1059615. After 48 hours, cells were collected, permeabilized and then stained with the mTOR monoclonal antibody and assessed by flow cytometry. Results were expressed as the mean ± SD from three experiments.

creased the PKM2protein level in MDA-MB 231 cells, when treated with GSK1059615 (p= 0.02). However, when cells were treated with the same mTOR inhibitor in 1g/L and 4.5g/L of glucose, the amount of PKM2 protein did not significantly change. In MCF-7 cells, glucose restriction, independly, decreased PKM2 protein level. However, GSK1059615 had an additional effect, by approximatively 60%, 30% and 50% in cells cultured in 0, 1 and 4.5g/L of glucose respectively.

Effect of glucose restriction and GSK1059615 treatment on the mTOR protein level

The effect of glucose restriction and GSK1059615 treatment was also studied on mTOR expression since it is known that mTOR is activated in most tumor cells. In addition, mTOR is an important effector of metabolic adaptation and the Warburg effect through its regulation of PKM2 and other glycolytic enzymes in normoxic conditions. The data showed (Table 1) that mTOR expression decreased in MDA-MB 231 by 3.9 fold only when cells were deprived of glucose and treated with 20 nM GSK1059615 However in MCF-7 cells, mTOR expression decreased by 4.5, 1.2 and 2.3 in cells cultured in 0, 1 and 4.5g/L of glucose respectively.

Effect of GR on lactate secretion and cellular glucose consumption

To examine whether reduced expression of the PKM2 isoform could reverse the Warburg effect, i.e., decrease lactate secretion and glucose consumption, we measured the concentration of secreted lactate and glucose in the medium of MDA-MB 231 and MCF-7 cells incubated at different glucose concentrations. Figure 2A shows that MDA-MB 231 cells incubated in DMEM and 1 g/L glucose secreted less lactate (P = 0.0125), and this reduction (approximately 80%) was more significant when the cells were starved of glucose (P =0.0001). Similarly, MDA-MB 231 cells increased glucose consumption when the glucose concentration was reduced to 1 and 0 g/L (P <0.01) (Figure 2C).

In MCF-7 cells, glucose deprivation reduced lactate secretion by 80% (P =0.0001) but tripled glucose

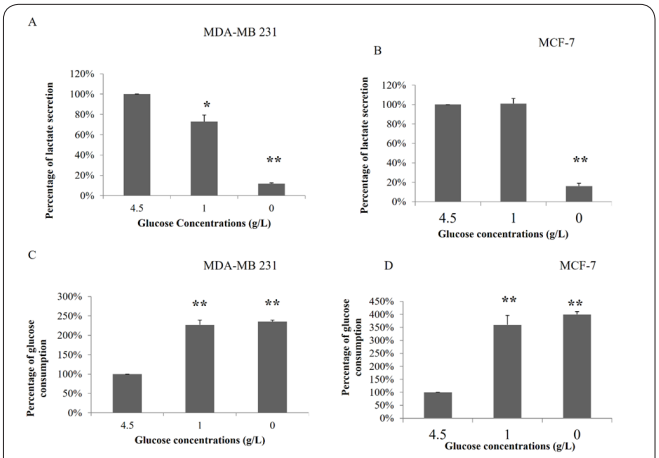

Figure 2. The effect of GR on lactate secretion and glucose uptake in MDA-MB 231 and MCF-7 cells. Cells were pre-incubated for five days in medium with various glucose concentrations and seeded in a six-well plate at a density of 200,000 cells/well. After a 24 h adhesion period, the concentration of lactate secreted into the culture medium was measured using a colorimetric assay. The optical density (O.D.) of the colored product was read at 505 nm (Figures 2 A&B). Similarly, the amount of glucose consumed was measured using the Trinder method, which generates a colored complex. Absorbance was measured at 500 nm and was proportional to the glucose concentration in the culture medium (Figures 2 C&D). The bars represent values of three independent experiments (* p <0.05, ** p <0.01). The percentage of glucose consumption is relative to the glucose consumption of cells cultured in medium with 4.5g/L.

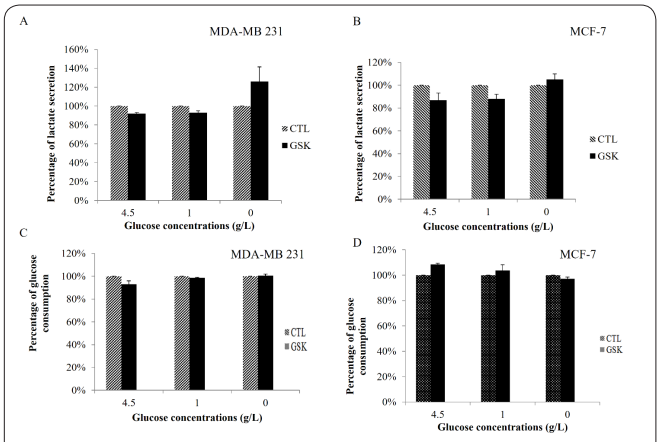

Figure 3. The effect of GSK1059615 treatment on lactate secretion and glucose consumption in MDA-MB 231 and MCF-7 cells. Cells were pre-incubated for five days with different amounts of glucose, seeded in a six-well plate at a density of 200,000 cells/well and treated after adhesion with 20 nM GSK1059615 for 48 h. After treatment, the concentration of secreted lactate was measured using a colorimetric assay, and the O.D. of the colored product was read at 505 nm. Similarly, the glucose consumption was measured using the Trinder method, which generates a colored complex whose absorbance at 500 nm is proportional to the glucose concentration in the culture medium. Figure 3 shows the concentration of secreted lactate (A, B) and glucose consumed (C, D) in MDA-MB 231 and MCF-7 cells. The bars represent values of three independent experiments. The percentage of glucose consumption is relative to the glucose consumption of cells not treated with GSK105915.

consumption (P = 0.0041) as shown in Figure 2B and 2D, respectively. However, the association of lactate secretion with significant increase in glucose consumption (P =0.0016) did not change between cells incubated

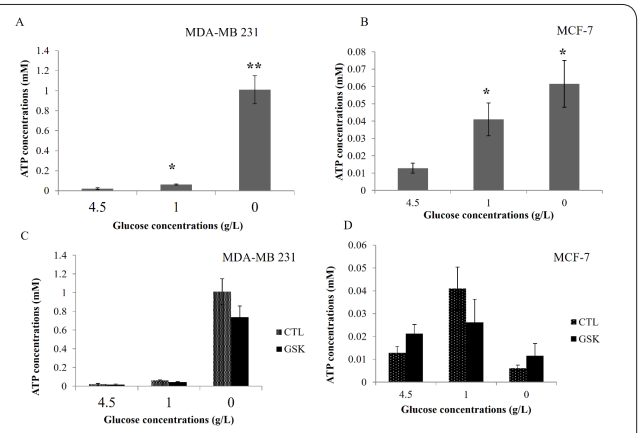

Figure 4. The effect of GR on intracellular ATP production in MDA-MB 231 and MCF-7 cells. MDA-MB 231 and MCF-7 cells were incubated with different amounts of glucose in the abscence (Figures 4 A&B) or presence of 20 nM GSK1059615 (Figures 4 C&D). After 48 h of treatment, 10^6 cells were subjected to an enzymatic colorimetric assay. The O.D of the colored product was measured at 570 nm using a spectrophotometer and was proportional to the concentration of intracellular ATP. The bars represent values of three independent experiments (* $p < 0.05$, ** $p < 0.01$).

in DMEM with 1 g/L and those incubated with 4.5 g/L glucose.

Effect of GSK1059615 treatment on lactate secretion and glucose consumption

Because the mTOR kinase positively regulates expression of inactive PKM2, we inhibited mTOR using a specific inhibitor, GSK1059615, and examined the efficacy of this inhibitor under glucose-restricted conditions.

As shown in Figure 3, GSK1059615 does not significantly modulate lactate secretion and glucose uptake in MDA-MB 231 or MCF-7 cell lines.

Effect of GR on intracellular ATP level

We measured intracellular ATP concentration after treatment with 20 nM GSK1059615 under glucose-restricted conditions. As shown in Figure 4, MDA-MB 231 cells increased ATP concentration 3-fold and 50-fold when incubated in medium containing 1 g/L ($P = 0.0192$) and 0 g/L glucose ($P = 0.0189$), respectively.

In MCF-7 cells, ATP concentration increased approximately 3-fold when cells were incubated with 1 g/L glucose ($P = 0.0371$) and 5-fold when deprived of glucose ($P = 0.02$).

Treating cells with 20 nM GSK1059615 did not change intracellular ATP levels in either cell line (Figure 4C and 4D).

Discussion

The mTOR serine/threonine kinase regulates cell growth, survival and protein synthesis (19). Recently, it has been demonstrated that mTOR regulates aerobic glycolysis in cancer cells and promotes tumor growth through up-regulation of the PKM2 protein (20).

PKM2 is the primary enzyme present in embryonic tissues, proliferating adult cells and tumor cells (21). PKM2 supports tumor growth through its two functions: acting as a glycolytic enzyme in the cytosol and acting as a protein kinase to enhance the transcription of many target proteins in the nucleus (22). The glycolytic and the transcriptional activity of PKM2 are controlled by allosteric regulation, its oligomeric state, post-translational modification and intracellular localisation (23).

Our study shows a link between mTOR, PKM2 and glucose availability. Glucose promotes tumor growth through various mechanisms. Increased glucose levels stimulate the IGF-1R/PI3K/Akt/mTOR proliferation pathway, which in turn strongly regulates the transcription factors c-Myc and HIF-1α (24). A recent study demonstrated that mTOR up-regulates PKM2 expression through the transcriptional activation of HIF-1 and c-Myc-regulated heterogeneous nuclear ribonucleoproteins that regulate PKM2 gene splicing (20). Similarly, PKM2 and HIF-1α can form a positive feedback loop that regulates the expression of HIF-1α target genes, favoring metabolic reprogramming and angiogenesis (17). Our results indicate a relative high expression of the PKM2 isoform in MCF-7 cells incubated in 4.5 g/L of glucose. This can be explained by a recent study that showed that PKM2 could be acetylated on lysine 305, especially at high glucose concentration. This acetylation reduces the enzymatic activity of PKM2 and promotes the accumulation of the inactive isoform. This post-translational regulation promotes tumor growth by stimulating the accumulation of metabolites for macromolecule synthesis (25). Another hypothesis that may explain the high expression of the PKM2 in this condition (incubation with 4.5 g/L glucose) is the ability of glucose to stimulate the PI3K/Akt/mTOR pathway. However, glucose restriction did not have a significant effect on PKM2 level, in MDA-MB-231 cells, which are triple negative breast cancer cells (TNBC). In fact, recent evidence indicates that c-Myc activates glucose metabolism in TNBC cells through direct repression of thioredoxin-interacting protein (TXNIP). TXNIP is a potent negative regulator of glucose uptake, aerobic glycolysis, and glycolytic gene expression; hence, its repression by c-Myc gives an alternate route to c-Myc–driven glucose metabolism. Furthermore, nuclear PKM2 induces expression of c-Myc though the activation of β-catenin. Thereby the expression of three nuclear PKM2-dependent glycolytic enzymes is upregulated, including glucose transporters 1 (GLUT1), lactate dehydrogenase A (LDHA) and PTB-dependent PKM2 (26). TXNIP suppression increases glucose uptake and drives a dependence on glycolysis. Supporting the biological significance of the reciprocal relationship between c-Myc and TXNIP, a $Myc_{high}/TXNIP_{low}$ gene signature correlates with decreased overall survival in breast cancer. The $Myc_{high}/TXNIP_{low}$ gene signature is associated with poor clinical outcome only in TNBC, not in other breast cancer subclasses. Mutation of TP53, which is a defining molecular feature of TNBC, enhances the correlation between the $Myc_{high}/TXNIP_{low}$ gene signature and death from breast cancer (27).

According to our results, high level of PKM2 was associated with activated mTOR level in both cell lines. When glucose and other nutrients are available, mTOR induces the expression of PKM2 via the induction of HIF-1α and c-Myc transcription factors (28).The role of glucose and other nutrients in regulating tumor growth has been frequently studied. De Lorenzo and colleagues studied a breast cancer model of mice fed a low caloric

diet and demonstrated a significant impairment of tumor growth and pulmonary metastasis. Caloric restriction (CR) also reduced angiogenesis by decreasing vascular endothelial growth factor (VEGF) levels in mice serum. Similarly, CR induced a reduction in expression levels of transforming growth factor-β (TGF-β), which is associated with lung metastasis, and metabolic markers such as insulin growth factor (IGF-1) (29). More recently, it has been demonstrated that the CR is a new therapeutic intervention that enhances the cytotoxic effects of radiation therapy in triple-negative breast cancer (30). Mice injected with triple-negative breast cancer cells and treated with a combination of radiotherapy and CR responded better to treatment, had less proliferative and more apoptotic tumors and had decreased expression of the molecular effectors of the IGF-R/PI3K /Akt/mTOR pathway.

Our results also showed that cellular glucose deprivation decreases lactate secretion but increases glucose consumption and ATP production in MDA-MB 231 and MCF-7 cells. These observed phenomena oppose the Warburg effect and stimulate partial oxidative phosphorylation. It seems that glucose restriction limits the metabolic flexibility of these tumor cells. This hypothesis supports the abundance of research that has focused on developing agents that reduce glucose bioavailability in the tumor cell microenvironment. The most important is metformin, a drug that treats type II diabetes by reducing glucose production by the liver and enhancing cellular insulin sensitivity. In this context, epidemiological studies have shown that patients with diabetes treated with metformin have improved survival rates and reduced incidence of several cancers, especially breast cancer (31). At the molecular level, metformin activates adenosine monophosphate protein kinase (AMPK), which acts as a metabolic stressor and inactivates mTOR and S6K kinases in breast cancer. Due to its ability to reduce insulin levels in serum, metformin is a therapeutic agent in the treatment of non-diabetic patients with breast cancer. In hormone-dependent breast cancer, metformin reduces the expression of cyclin D and E2F transcription factors and inactivates Her-2 kinase and its effectors; while, in triple-negative breast cancer cells, metformin reduces cell proliferation (32). Moreover, metformin induces extrinsic and intrinsic apoptotic pathways following a decrease in epithelial growth factor receptor (EGFR) oncogene-stimulated signaling (33). Until now, no direct link has been established between reduced glucose availability and the level of the PKM2 isoform. Most research focused on the expression of the mTOR kinase under physiological stress conditions, as this kinase regulates and dictates the energy metabolism of the cell. Our results show that the amount of mTOR protein increased in 1g/L of glucose comparing to 4.5g/L of glucose in MDA-MB-231 and in MCF-7 cell lines. Then, when glucose restriction was more pronounced (0g/L of glucose), a decreasing of mTOR level was observed. It seems that cells acquired a resistance to glucose restriction when glucose concentration was decreased from 4.5g/L to 1g/L. This observation could be explained by two hypothesis: first, mTORC1 inhibition leads to up-regulation of receptor tyrosine kinases (RTKs or substrates) such as platelet-derived growth factor receptors (PDGFRs) and insulin receptor substrate 1 (IRS-1), resulting in increased PI3K-dependent AKT phosphorylation at Ser473. Second, mTORC1 inhibition leads to increased mitogenactivated protein kinase (MAPK) signaling via PI3K-Ras activation. In addition to activating PI3K-AKT and MAPK signaling, mTOR inhibition by rapamycin can also induce MYC phosphorylation and accumulation in colorectal cancer cells(21). Taken together, PI3K-mTOR inhibitors such as rapamycin, BEZ235, and GDC0941 lead to acquired resistance to PI3K-mTOR-targeted therapy in cancer cells.by inducing either PI3K-dependent or MYC-dependent mechanisms (34) . Based on our results, we can suggest that mTOR inhibition by GSK 1059615 is reinforced by glucose restriction. However, several studies have revealed that the resistance of cancer cells to a variety of anticancer drugs can increase via upregulation of autophagy. When mTORC1 is inhibited under various stressful conditions, such as starvation and organelle damage, autophagy is enhanced. mTORC1 is regulated by AMP-activated protein kinase (AMPK), and inhibition of mTORC1 and increased AMPK induces the autophagic process (28). Although some autophagy modulators, such as rapamycin and chloroquine, are used to regulate autophagy in anticancer therapy, since this process also plays roles in both tumor suppression and promotion, the precise mechanism of autophagy in cancer is not clear (35).

Because the Warburg effect is an essential phenomenon that supports cell growth and division, our study provides a novel framework to understand the potential role of glucose restriction in reducing the Warburg effect via the modulation of mTOR and PKM2 in breast cancer cell lines.

Glucose restriction plays an important role in the reduction of the Warburg effect by regulating the amounts of mTOR and PKM2 kinases. Similarly, cellular deprivation of glucose may,in some part, improve inhibition of the mTOR kinase. GR is a simple therapy that involves a lifestyle change to decrease glucose consumption. This is a promising step for the treatment of cancer as a primary therapy or as an adjuvant therapy coupled to chemotherapy or radiotherapy.

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Ethics approval and consent to participate
This project was approved by the Ethical Committee of Saint-Joseph University and Hotel-Dieu de France.

Consent for publication
Not applicable.

Availability of data and materials
The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing of interest
The authors declare that they have no competing inte-

rests.

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Authors' contributions
RT participated to the study design and carried out the cell cultures ,the western blot and manuscript revision. LW participated to the study design and cell cultures and western blot and manuscript revision. RS, RH and IR helped to interpret the results and drafted the manuscript. GH conceived of the study and participated to its design and coordination and helped to draft and correct the manuscript.

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