

Loss of function of PTEN alters the relationship between glucose concentration and cell proliferation, increases glycolysis, and sensitizes cells to 2-deoxyglucose

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A B S T R A C T

PTEN loss of function enhances proliferation, but effects on cellular energy metabolism are less well characterized. We used an inducible PTEN expression vector in a PTEN-null glioma cell line to examine this issue. While proliferation of PTEN-positive cells was insensitive to increases in glucose concentration beyond 2.5 mM, PTEN-null cells significantly increased proliferation with increasing glucose concentration across the normal physiologic range to ~10 mM, coinciding with a shift to glycolysis and “glucose addiction”. This demonstrates that the impact of loss of function of PTEN is modified by glucose concentration, and may be relevant to epidemiologic results linking hyperglycemia to cancer risk and cancer mortality.

Keywords:

Glucose
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1. Introduction

PTEN is a tumor suppressor protein with phosphatase activities [1,2]. Loss of function of PTEN is frequent in neoplasia and is associated with poor prognosis [3]. Studies of PTEN heterozygous mice demonstrate that PTEN is involved in regulation of energy metabolism at the whole organism level, as haploinsufficiency confers insulin hypersensitivity [4]. Effects of loss of function of PTEN on cellular proliferation and survival have been described in detail, but relatively little attention has been given to consequences of loss of PTEN function on cellular energetics.

There is evidence that transformed cells use glycolysis rather than oxidative phosphorylation to generate ATP, as originally proposed by Warburg [5,6]. The role of PTEN in

this process is not completely understood, and the use of an inducible PTEN expression vector to clarify the function of this phosphatase in regulation of cellular energy metabolism in neoplastic cells has not been reported, although there have been studies of the role of PTEN in glucose metabolism in untransformed cells. For example, it has been previously suggested by applying siRNA methods to 3T3-L1 adipocytes that PTEN can suppress the effects of insulin signaling on glucose transport through the PI3K pathway [7], although others [8], using the same cells transfected with a PTEN phosphatase-dead mutant suggested that PTEN may not play a major role in the regulation of glucose transport activity.

We used the PTEN-null human glioma cell lines U251 and U87 transfected with a tetracycline-inducible PTEN expression vector system [9] to study the role of PTEN in cellular energy metabolism and glucose dependency of transformed cells.

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2. Materials and methods

2.1. Cell culture

The PTEN-null human glioma cell lines, U251 and U87, transfected with a tetracycline-inducible PTEN expression vector system were kindly obtained from Dr. M.M. Georgescu (University of Texas) and cultured in DMEM (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Wisent Inc., St. Bruno, QC, Canada), 0.5 mg/ml G418 (Invitrogen), and 10 µg/ml blasticidin (InVivoGen, San Diego, CA). Under these conditions, the expression of PTEN had been previously shown to be non-leaky [9]. PTEN expression was induced by the addition of 1 µg/ml doxycycline (Sigma Chemical, St. Louis, MO). Cells were kept at 37 °C and 5% CO₂. Other cell culture reagents were purchased from Invitrogen.

2.2. Cell treatments

Cells were plated in 96-well plate (3500 cells/well), 12-well plate (10⁵ cells/well), 6-well plate (3 × 10⁵ cells/well), or in 10-cm petri dish (1.5 × 10⁶ cells) for 24 h. Medium was changed for fresh medium containing or not doxycycline for another 24 h. The day after, the medium was replaced by serum free medium (SFM), with or without doxycycline supplemented or not with insulin (850 nM, Cell Sciences, Canton, MA). Forty-eight hours after treatment, supernatant was collected and stored at -80 °C until use and cells were trypsinized and counted (Trypan blue exclusion) or were used to prepare cell lysates. Other treatments included: (1) increasing concentrations of insulin added to SFM; (2) to analyze the effect of glucose, cells were cultured in SFM without glucose to which various concentrations of glucose were added; and (3) the effect of 2-DG, an analogue of glucose, was assayed at different concentrations using SFM containing 16 mM of glucose. For these experiments, treatments were applied for 72 h. All experiments were performed at least twice and each condition was in triplicates or four replicates.

2.3. Western blotting

After 48 h exposure to treatments, cells were processed as described [10]. Briefly, U251 and U87 cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P40, 0.05% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Complete tablets, Roche, Laval, QC, Canada)). The protein concentration in the supernatant fraction was determined and the extracts were stored at -80 °C. Proteins (50 µg) were resolved electrophoretically on denaturing SDS-polyacrylamide gels (10%), transferred to nitrocellulose membranes, and probed with the following antibodies overnight at 4 °C: anti-PTEN, anti-hypoxia-inducible factor-1 alpha (HIF-1α, anti-Glut1 (Santa-Cruz Biotechnologies, Santa Cruz, CA), anti-total AKT, anti-phospho-AKT^(Ser473), anti-phospho-AKT^(Thr308), anti-hexokinase II (HKII), anti-p53, and anti-β-actin (Cell Signalling Technology, Beverly, MA) antibodies. The posi-

tion of the proteins was visualized with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin antibodies (ECL, Amersham, Baie d'Urfé, QC, Canada). Experiments were repeated at least twice. Band intensity was evaluated by densitometry using Scion Corporation software (version Alpha 4.0.3.2, Frederick, MD).

2.4. Cell proliferation assay

We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical) assay to determine the effects of insulin, glucose, or 2-deoxyglucose (2-DG) on cell growth (cell viability) of U251 and U87 cells expressing or not PTEN. After appropriate treatment time (72 h), MTT was added to a final concentration of 1 mg/ml, and the reaction mixture was incubated for 3 h at 37 °C. The resulting crystals were dissolved in 0.04% HCl in isopropanol and the absorbance was read at 562 nm. Four replicates were used for each treatment, and each experiment was repeated twice. Cell proliferation in presence of various concentrations of glucose was also evaluated by 0.4% Trypan blue exclusion cell counting. Triplicates were used for each conditions and the experiment was repeated twice.

2.5. Cell cycle analysis by flow cytometry

After 72 h of treatment, floating and adherent cells were collected, washed, and fixed in 100% ethanol at -20 °C overnight. Cell cycle analysis was performed by incubating cells in propidium iodide buffer (PBS, 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml ribonuclease A, and 50 µM propidium iodide) for 30 min at room temperature, and then the cell cycle distribution was determined by flow cytometry with a FACSCalibur Instrument (Becton-Dickinson, Franklin Lakes, NJ) using CellQuest software (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ).

2.6. Glucose consumption assay

Media samples were collected and glucose consumption was measured using a colorimetric assay described previously [11]. Briefly, on the day of the assay, 10 µl of medium samples were mixed with 435 µl of H₂O, 500 µl of the buffer/chromophore reagent (0.8 M sodium Phosphate buffer, pH 6.0, 10 mM 4-aminoantipyrine, and 10 mM N-ethyl-N-sulfopropyl-m-toluidine), 5 µl of 540 U/ml horseradish peroxidase, and 50 µl of 310 U/ml glucose oxidase. A standard curve was made using D-glucose at concentrations varying between 0.05 and 25 mM. As a negative control, glucose oxidase was omitted and replaced by H₂O. The reactions were incubated for 45 min in the dark, and then the absorbance was read at 562 nm. Each condition was in duplicate and the experiment was performed three times. We found that results of this assay were completely consistent with results obtained with a tritiated glucose uptake assay [8,12] (data not shown).

2.7. Lactate assay

Media samples were collected and lactate was measured using a colorimetric kit according to the manufacturer's instructions (BioVision Research Products, Mountain View, CA). Each condition was in duplicate and the experiment performed twice.

2.8. Measurements of oxygen consumption and extracellular acidification rates

U251 cells were pre-treated or not with doxycycline for 24 h. Then, cells were trypsinized and seeded in XF 24-well plates at 2.3×10^4 cells/well for 20 h. At that time, the medium was changed to unbuffered serum-free DMEM and oxygen consumption and extracellular acidification rates were measured using a Seahorse Bioscience XF24 instrument (North Billerica, MA) as previously described [13]. Following the analysis, cells were trypsinized and counted using a Z2 Coulter counter (Mississauga, ON, Canada). Data were normalized with the number of cells. The experiment was performed twice and each condition had four replicates.

2.9. Statistics

Data were analyzed with one-way ANOVA (for the proliferation and glucose consumption assays), two-way ANOVA (cell cycle analysis and cell counting) with post hoc test for multiple comparisons (LSMEANS statement with Bonferroni correction), or with unpaired Mann Whitney U Test (non-parametric) for comparison between two groups (for lactate production assay). The Wilcoxon-Rank test was used for the difference of paired values between the two groups for oxygen consumption and extracellular acidification rates. Statistical significance was evaluated using GLM Procedure by Statistical Analysis System software, version 9.1.3 (SAS Institute, Cary, NC), with p values ≤ 0.05 considered significant.

3. Results

3.1. Validation of the experimental system

To verify the performance of our experimental system, we examined the protein levels of PTEN and phosphorylation of selected signaling molecules in the presence or absence of doxycycline. Forty-eight hours exposure to doxycycline in SFM was sufficient to induce PTEN expression in U251 cells (Fig. 1A). Since PTEN is a negative regulator of AKT, and AKT is a central player in insulin and glucose pathways, it was important to confirm the relationship between the PTEN expression and AKT phosphorylation. The expression of PTEN had no effect on total AKT levels (Fig. 1B). However, as expected, we observed that AKT activation was increased in PTEN-null cells compared with PTEN-positive cells, and that the presence of insulin further increased AKT^{Ser473} (Fig. 1C) and AKT^{Thr308} phosphorylation (Fig. 1D). Forced expression of PTEN in our experimental system was also associated with decreased levels of HIF-1 α (Fig. 1E) and hexokinase II (HKII) (Fig. 1F), and increased levels of p53 (Fig. 1G). There were no significant changes in the protein levels of glucose transporter (Glut) 1 (Fig. 1H) nor of Glut 4 (data not shown). The presence of insulin increased the levels of HIF-1 α and HKII, both in PTEN-positive and PTEN-null cells, while had little effects on p53 and Glut1 levels. These results are in agreement with recent reports (reviewed in [14–16]). We also observed the same pattern with the cell line U87 (data not shown).

3.2. The effect of loss of PTEN on proliferation varies according to glucose concentration

When cultured in SFM for a period of 72 h, PTEN-positive cells were more sensitive to insulin stimulation than PTEN-null cells ($p < 0.0001$, Fig. 2A); the percent growth stimulation by insulin was increased by only 32.0% for the PTEN-null cells compared with an increase of 117.9% for the PTEN-positive cells. However, as expected, PTEN-null cells proliferated at a higher rate than PTEN-positive cells regardless of insulin concentration.

As shown in Fig. 2B and C, PTEN-null cells, but not PTEN-positive cells, exhibited accelerated proliferation when the glucose concentration was increased between 2.5 and 10.0 mM ($p < 0.0001$). As glucose concentration was elevated, proliferation of PTEN-null cells increased to 534.5% of control levels and the dose response curve showed a plateau at ~ 7 mM. In contrast, PTEN-positive cell proliferation increased only to 334.0% of control, and a plateau was observed at a glucose concentration of ~ 2.5 mM. The presence of PTEN was, as expected, associated with reduced proliferation in both low glucose and high glucose conditions, although the magnitude of this effect was much greater at 15 mM than at 2.5 mM. Thus, the increase in proliferation associated with loss of function of PTEN varies with glucose concentration. U87 cells followed the same pattern as U251 cells in presence of increasing concentrations of glucose: PTEN-null U87 cell proliferation plateaued ~ 5 mM glucose while for PTEN-positive cells, proliferation plateaued at 2.5 mM glucose (data not shown).

3.3. Effects of PTEN loss on cell cycle are influenced by glucose concentration

The above results were extended by the flow cytometry studies of cell cycle shown in Fig. 2D. PTEN expression was associated with increased percentage of cells in sub-G₀/G₁ compared to PTEN-null cells at both low and high glucose concentration (2.5 mM glucose: $12.0\% \pm 0.3$ vs. $5.1\% \pm 0.4$, $p = 0.0017$; 15 mM glucose: $10.5\% \pm 1.8$ vs. 2.0 ± 0.2 , $p = 0.0003$) as expected [17], and also with reduced percentage of cells in S-phase (2.5 mM glucose: $4.9\% \pm 0.1$ vs. $8.1\% \pm 0.2$, $p < 0.0001$; 15 mM glucose: $5.4\% \pm 0.1$ vs. 10.0 ± 0.3 , $p < 0.0001$). The percentages of cells in G₀/G₁ and G₂/M phases were not significantly different between PTEN-null and PTEN-positive cells both at low and high glucose concentration. There was no statistically significant difference in percentage of cells in sub-G₀/G₁ phase when increasing glucose concentration for both PTEN-null and PTEN-positive cells. Interestingly, the percentage of PTEN-null cells in S-phase increased significantly when glucose concentration was raised from 2.5 mM to 15 mM ($8.1\% \pm 0.2$ – $10.0\% \pm 0.3$, $p < 0.0001$), while the percentage of PTEN expressing cells in S-phase was relatively unaffected by the change in glucose concentration, again indicating that the impact of loss of function of PTEN on proliferation is modified by glucose concentration.

3.4. PTEN loss is associated with increased sensitivity to 2-deoxyglucose (2-DG)

2-DG is a glucose analogue that acts as a competitive inhibitor of glucose metabolism [18–20]. PTEN-null cells cultured in increasing concentrations of 2-DG (at a constant concentration of glucose of 16 mM) were more sensitive to 2-DG than PTEN-positive cells ($p < 0.0001$, Fig. 2E). PTEN-null cell growth was inhibited by 52.6% at 16 mM 2-DG compared with the control without 2-DG, whereas growth of PTEN-positive cells was inhibited by only 9.1% at 16 mM 2-DG. The increased sensitivity to 2-DG induced growth inhibition suggests that loss of function of PTEN is associated not only with well-described increased proliferation and survival, but also with “glucose addiction”.

3.5. Influence of PTEN loss on glycolysis

To examine in more detail whether PTEN has a role in regulating glycolysis in cancer cells, we measured glucose consumption. PTEN-null cells consumed almost twice the amount of glucose than PTEN-positive cells (17.2 ± 2.0 vs. 8.8 ± 1.5 mM/million cells/48 h; $p < 0.0001$, Fig. 3A). These results were confirmed with results obtained with the tritiated 2-DG uptake assay method (data not shown). The presence of insulin did not significantly influence glucose consumption, consistent with the view that although cancer cells often express insulin receptors and can be mitogenically stimulated by insulin (for example [21], reviewed in

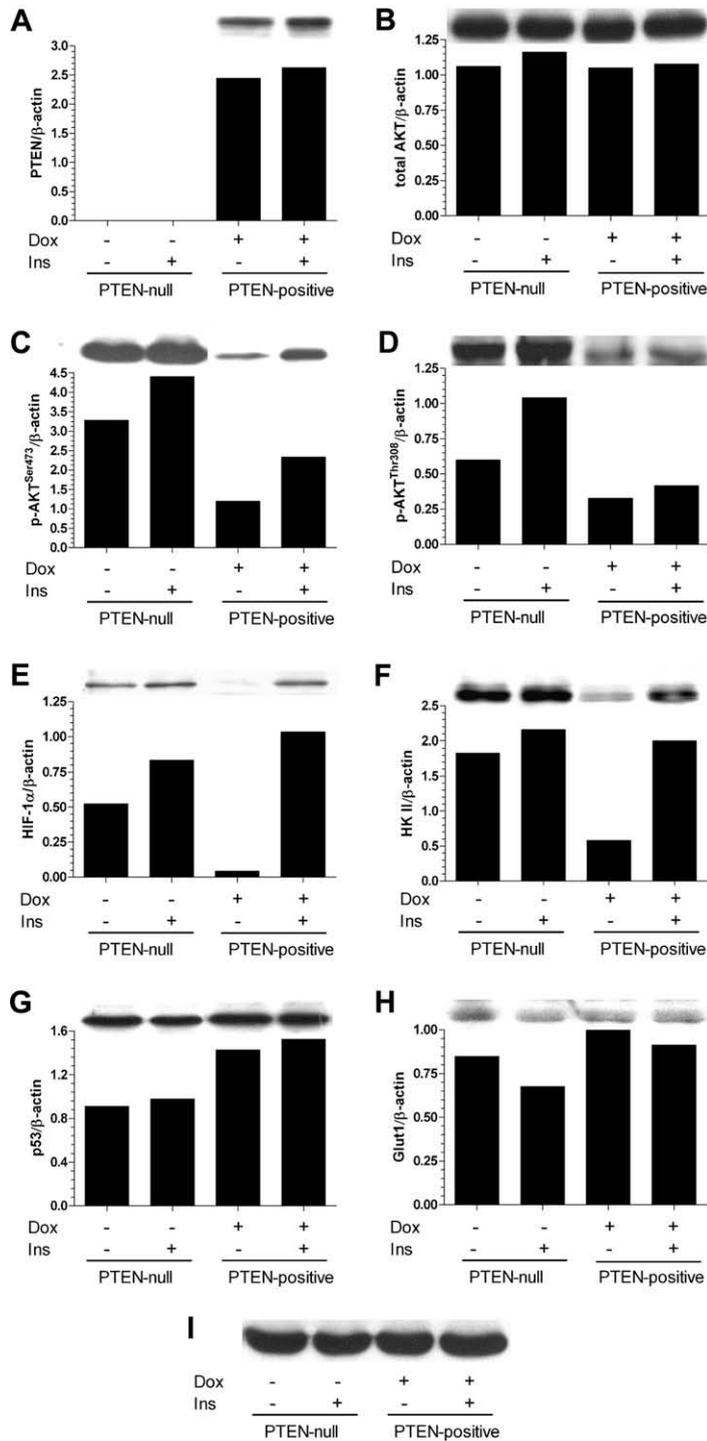


Fig. 1. Signaling varies according with U251 PTEN status. For each condition, cell lysates from U251 PTEN-null and U251 PTEN-positive (induced by 1 μ g/ml doxycycline) cells with or without insulin (Ins, 850 nM) were prepared and analyzed by Western blot using specific antibodies: (A) anti-PTEN; (B) anti-total AKT; (C) phospho-AKT^{Ser473}; (D) phospho-AKT^{Thr308}; (E) anti-HIF-1 α ; (F) anti-HKII; (G) anti-p53; (H) anti-Glut1; and (I) anti- β -actin. Levels of each protein were evaluated by densitometry. Bars indicate results of densitometry and in each case represent the ratio of the signal of the antibody of interest to the signal of a β -actin loading control. Data shown are representative of at least two independent experiments.

[22]), their glucose uptake is not necessarily insulin-regulated. We also observed that PTEN-null U251 cells produced more than three times the amount of lactate of U251 cells with forced PTEN expression

(35.9 ± 4.8 vs. 10.7 ± 2.3 mM/million cells/48 h; $p < 0.0001$, Fig. 3B), demonstrating increased glycolysis. As another means of confirming these results, we also determined cellular extracellular acidification rates (ECAR)

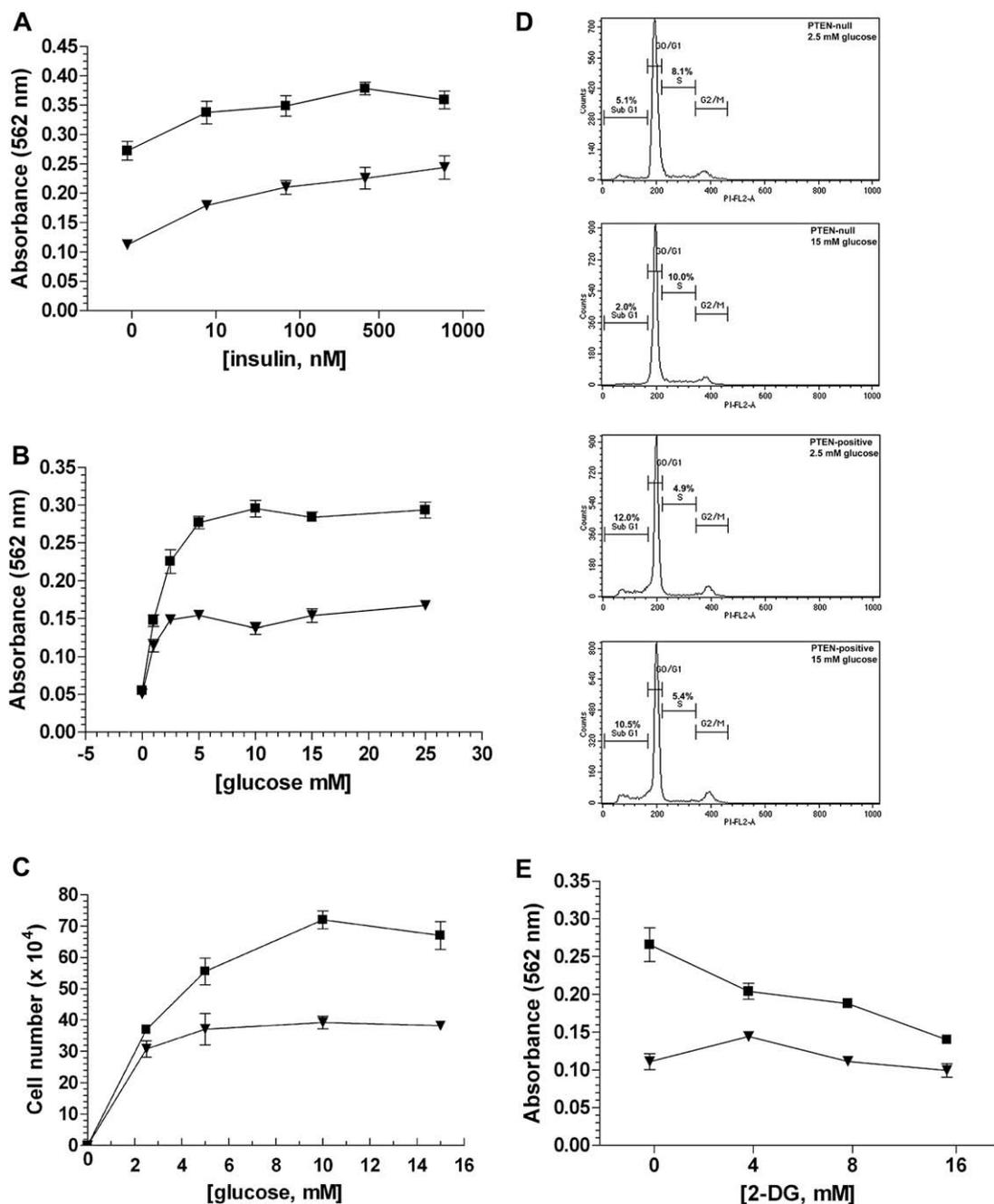


Fig. 2. Effects of PTEN expression, insulin, glucose, and 2-DG on U251 cell proliferation. Twenty-four hours after plating, the medium of U251 cells either exposed or not to doxycycline (Dox, 1 $\mu\text{g}/\text{ml}$) was changed to (A) SFM supplemented with increasing concentrations of insulin; (B and C) glucose-free SFM was supplemented with increasing concentrations of glucose; (D) glucose-free SFM was supplemented with 2.5 mM or 15 mM glucose; and (E) SFM containing 16 mM glucose and supplemented with various concentrations of 2-DG. Cells were incubated for another 72 h, in presence or not of doxycycline, at which point a cell proliferation assay (MTT) was performed (A, B, and E), cell proliferation was determined by Trypan blue exclusion cell counting (C), or floating and adherent cells were collected and their cell cycle was determined by PI staining using flow cytometry (D). For A–C, and E, data are presented as mean \pm SE from two independent experiments done in quadruplicates. Square: PTEN-null cells, inverted triangle: PTEN-positive cells; $p < 0.0001$, between PTEN-null cells and PTEN-positive cells for A, B, and E. (C) $p = 0.0167$ at 5 mM glucose and $p < 0.0001$ at 10 and 15 mM glucose between PTEN-null and PTEN-positive cells. (D) In sub-G₀/G₁ phase: $p = 0.0017$ between PTEN-null and PTEN-positive cells in 2.5 mM glucose; $p = 0.0003$ between PTEN-null and PTEN-positive cells in 15 mM glucose; In S phase: $p < 0.0001$ between PTEN-null and PTEN-positive cells in 2.5 mM glucose, between PTEN-null and PTEN-positive cells in 15 mM glucose, and between PTEN-null cells in 2.5 mM glucose vs. in 15 mM glucose.

which is a measure for glycolysis, lactate production and anaerobic metabolism, of both PTEN-null and PTEN-positive U251 cells. PTEN-null

cells had a basal ECAR significantly higher than for PTEN-positive cells (1.99 ± 0.12 vs. 1.31 ± 0.07 pH/min/ 10^6 cells, $p = 0.0048$) (Fig. 3C). The

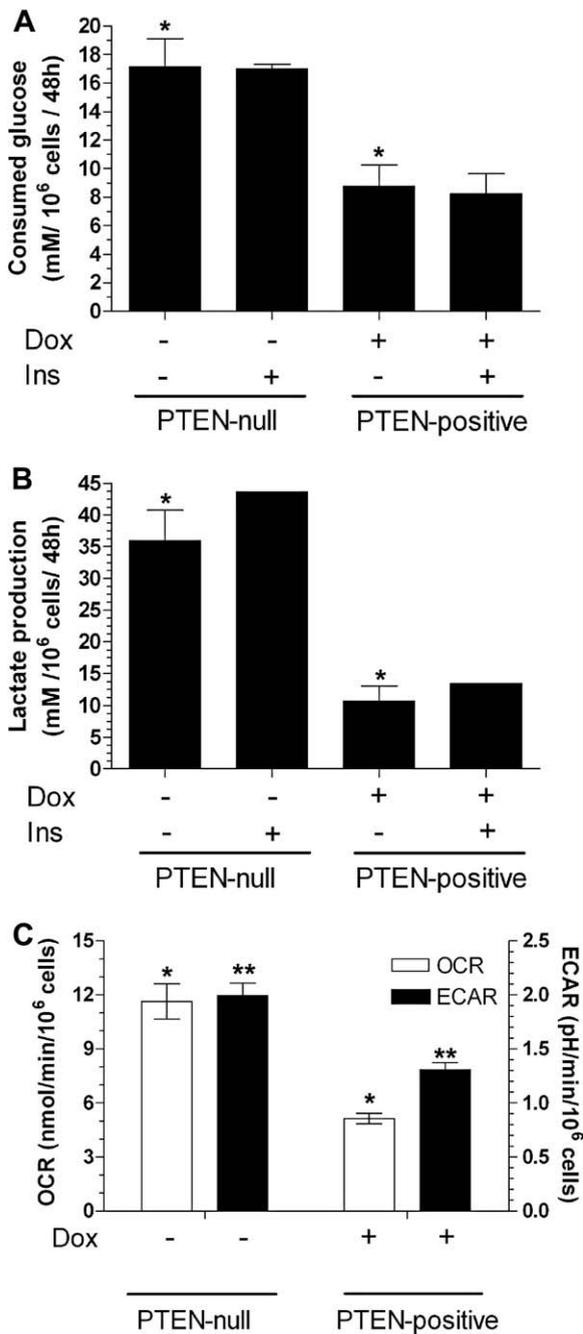


Fig. 3. PTEN expression influences glucose consumption, lactate production, and oxygen consumption by U251 cells. Twenty-four hours after plating, the medium of U251 cells either exposed or not to doxycycline was changed to (A and B) SFM with or without insulin (Ins, 850 nM): After 48 h of treatment, culture medium was collected from each well and was analyzed by (A) a glucose consumption assay, *: $p < 0.0001$; or (B) a lactate production assay, *: $p = 0.0009$. Columns represent the mean \pm SE of two independent experiments done in duplicate. (C): U251 cells pre-treated or not with doxycycline were analyzed for basal oxygen consumption (OCR, *: $p = 0.0009$, white columns on left Y axis) or extracellular acidification rate (ECAR, **: $p = 0.0048$, black columns on right Y axis).

addition of 2-DG during the measurement decreased ECAR of PTEN-null cells by 46% of the baseline levels while this decrease was only 27% in

PTEN-positive cells. Interestingly, the lack of PTEN in U251 cells was associated with basal oxygen consumption rate (OCR) double that when PTEN was expressed (11.65 ± 0.98 vs. 5.14 ± 0.30 nmol/min/10⁶ cells, $p = 0.0009$) (Fig. 3C), suggesting that PTEN plays a role in both regulating glycolysis and aerobic mitochondrial oxidation.

4. Discussion

There is prior mechanistic evidence that AKT activation promotes glycolysis [15,16,23]. However, the important consequence of altered glucose dependency as a result of PTEN loss has not been described. The data demonstrate that variation in glucose concentration in the clinically relevant range of ~ 2.5 –10 mM [24] has little impact on PTEN-positive cells, but that high glucose levels facilitate rapid proliferation of PTEN-null cancers, and indeed are necessary for the maximal effects of loss of PTEN on proliferation.

Craig Thompson's laboratory showed that the glioblastoma cell line LN229 transfected with a myristoylated constitutive active form of AKT increased glucose consumption (consistent with increased glycolysis) without promoting cell proliferation or increasing oxygen consumption [23], which differs from some of our results. We observed that two glioblastoma cell lines, U251 and U87, had decreased proliferation, decreased glucose consumption, and decreased oxygen consumption when PTEN was expressed. These differences may be related to evidence that loss of function of PTEN is not physiologically equivalent to constitutive activation of AKT, in view of evidence that PTEN has functions beyond regulation of the AKT pathway [25]. Furthermore, other cellular systems with constitutive activity of AKT showed increased proliferation [26–28]. Furthermore, while increased glycolysis is a major source of energy for cancer cells, this does not mean that they necessarily have impaired oxidative phosphorylation [29]. In fact, among cancer cells, there is heterogeneity in energy production from oxidative phosphorylation [29–32]. We observed that proliferation (as assessed by cell number) differs little with PTEN status at low glucose concentrations, while at glucose concentration higher than 5 mM, loss of function of PTEN increases proliferation. This is consistent with the hypothesis that loss of function of PTEN in our model is permissive for increased proliferation, but requires increased glucose supply.

There is increasing experimental (for example [33,34]) and epidemiologic (for example [35–39]) evidence that cancer risk is higher and outcome is worse in patients with features of 'metabolic syndrome', which includes obesity, hyperinsulinemia, and hyperglycemia (reviewed in [22,40]). This has important public health implications, given the increased prevalence of obesity and hyperglycemia in affluent societies. Most speculation to date concerning the mechanism underlying this association (reviewed in [22]) has emphasized the potential role of insulin as a mediator: elevated insulin concentration (due to increased insulin secretion in the setting of metabolic syndrome-related insulin resistance in classic insulin target tissues such as muscle and fat) is hypothesized to stimulate insulin receptors present in cancer cells, activating the AKT path-

way. Our results suggest that elevation of glucose to the extent seen in clinical hyperglycemia can also act directly to increase proliferation of PTEN-null neoplastic cells, and motivate clinical studies to determine if hyperglycemia (or other markers of metabolic syndrome) differentially influences neoplastic behavior according to PTEN status. Furthermore, our results suggest that PTEN status predicts sensitivity to 2-DG and other agents under investigation as cancer treatments [18–20] that exploit glucose dependency.

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