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The PI3K/Akt Pathway Regulates Oxygen Metabolism via Pyruvate Dehydrogenase (PDH)-E1 α Phosphorylation

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Abstract

Inhibition of the PI3K/Akt pathway decreases hypoxia within SQ20B human head and neck cancer xenografts. We set out to understand the molecular mechanism underlying this observation. We measured oxygen consumption using both a Clark electrode and an extracellular flux analyzer. We made these measurements after various pharmacologic and genetic manipulations. Pharmacologic inhibition of the PI3K/mTOR pathway or genetic inhibition of Akt/PI3K decreased the oxygen consumption rate (OCR) *in vitro* in SQ20B and other cell lines by 30–40%. Pharmacologic inhibition of this pathway increased phosphorylation of the E1 α subunit of the pyruvate dehydrogenase (PDH) complex on Ser293, which inhibits activity of this critical gatekeeper of mitochondrial respiration. Expressing wild type PTEN in a doxycycline-inducible manner in a cell line with mutant PTEN led to an increase in PDH-E1 α phosphorylation and a decrease in OCR. Pre-treatment of SQ20B cells with dichloroacetate (DCA), which inhibits PDH-E1 α phosphorylation by inhibiting dehydrogenase kinases (PDKs), reversed the decrease in OCR in response to PI3K/Akt/mTOR inhibition. Likewise, introduction of exogenous PDH-E1 α that contains serine to alanine mutations, which can no longer be regulated by phosphorylation, also blunted the decrease in OCR seen with PI3K/mTOR inhibition. Our findings highlight an association between the PI3K/mTOR pathway and tumor cell oxygen consumption that is regulated in part by PDH phosphorylation. These results have important implications for understanding the effects PI3K pathway activation in tumor metabolism and also in designing cancer therapy trials that use inhibitors of this pathway.

Keywords

PI3K/Akt pathway; pyruvate dehydrogenase; oxygen metabolism

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Introduction

The PI3K/Akt/mTOR pathway is commonly activated in human cancers and plays a critical role in the development and maintenance of tumors (1). It has been implicated in multiple cellular processes involved in cell survival and growth including proliferation, adhesion, migration, invasion, and metabolism (2). For this reason, pharmacological companies have developed multiple drugs targeting this pathway (3–5). Some of these compounds have shown tolerable toxicity profiles in early stage trials and are being further investigated as single agents or in combination with other modalities.

The PI3K pathway has been found to have an important role in metabolism by increasing glucose uptake (6, 7). However, its effects on oxygen consumption have been less well studied. In the current study we investigated the effects of PI3K/mTOR inhibition on oxygen utilization and tumor hypoxia. Hypoxia is present in most solid tumors (8) and has been associated with resistance to therapy including radiation and chemotherapeutic agents (9–13). A number of strategies have been employed to reverse tumor hypoxia, such as increasing oxygen delivery to tumors using hyperbaric oxygen or carbogen (14, 15); however, these have met with limited success, partly due to the abnormal and leaky tumor vasculature and the consumption of oxygen by the tumor cells limiting its diffusion to regions distal to tumor vessels (16). An alternate means of decreasing hypoxia in tumors would be to decrease oxygen consumption by tumor cells. We and others have previously reported that tumor hypoxia can be reversed *in vivo* by agents that affect the PI3K/mTOR pathway (17–19). In investigating the molecular mechanism underlying this effect, we identified a novel link between PI3K/mTOR activation and phosphorylation (and inactivation) of pyruvate dehydrogenase (PDH), which catalyzes the conversion of pyruvate to acetyl CoA, thereby regulating mitochondrial respiration. Consequently, inhibition of the PI3K pathway would be predicted to lead to decreased oxygen consumption and concomitantly increased tumor pO₂. Our findings shed further light as to how the PI3K/mTOR pathway regulates cellular metabolism. They have important potential clinical implications in terms of using PI3K/mTOR inhibitors in combination with radiation to treat human cancers.

Materials and Methods

Chemicals

NVP-BEZ235 (referred to as BEZ235), NVP-BGT226 (referred to as BGT226), GDC-0068, and GDC-0980 were obtained from Selleck Pharmaceuticals (Houston, TX). These drugs were dissolved in DMSO at a stock concentration of 100 µM.

Cell growth

SQ20B and FaDu cells were obtained from American Type Culture Collection (Rockville, MD). SQ20B and FaDu head and neck squamous cell carcinoma cells were cultured in DMEM (4,500 mg/L glucose; Invitrogen, NY, USA) containing 10% fetal bovine serum (Atlanta Biologicals; NY, USA), penicillin (100 units/ml), and streptomycin (100 mg/ml; Life Technologies, Inc., Gaithersburg, MD) at 37°C in humidified 5%CO₂-95% air. U251-

PTEN and U251-C124S cells were obtained from Dr. Georgescu at MD Anderson Cancer Center (20). All 4 cells lines were authenticated by IDEXX RADIL (Columbia, MO).

Transfection of Cells with siRNA

Cells were transfected with ON-TARGET plus SMART pool siRNA (GE Dharmacon) against Akt-1 or PDH-E1 α . Briefly, cells were harvested and plated at a density of 200,000 cells per well in a six well plate and allowed to attach over night. The next day media was removed and cells were washed twice with PBS and re-fed with 1 ml of OPTI-MEM from Gibco. The six well plate was returned to the incubator for 1 hour before they were transfected. siRNA was mixed with Oligofectamine reagent (Invitrogen, NY) for 20 minutes before being added to the dishes.

Protein Extraction and Western Blot Analysis

Protein isolation and quantitation and Western blotting were performed as described previously (21). Antibodies directed against the following proteins were obtained from Cell Signaling Technology (Danvers, MA, USA): phospho-Akt (Ser473), Akt1, phospho-4E-BP1 (Ser 65), phospho-S6, pyruvate dehydrogenase (C54G1), β -actin, and PTEN. The following antibodies were obtained from Abcam (Cambridge, MA): pyruvate dehydrogenase E1 α subunit (phospho-S293), pyruvate dehydrogenase E1 α subunit (phospho-S232), pyruvate dehydrogenase E1 α subunit (phospho-S300), pyruvate dehydrogenase E2 subunit, pyruvate dehydrogenase E1 β subunit, pyruvate dehydrogenase E2/E3 subunit. The secondary antibody used for these blots was either a goat anti-mouse and goat anti-rabbit antibody from Thermo Scientific (Rockford, IL). Antibody binding was detected using an enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK).

Oxygen Electrode Measurements

Cells were treated with drug for 16 hours prior to being trypsinized and suspended in media (DMEM with 1% FBS, 1mM pyruvate, 1 mM glutamate, and 25 mM HEPES) and kept on ice until added to sealed chambers. An aliquot of the cell suspension was added to 3 ml of media in the glass chamber of the YSI magnetic stirring apparatus. Oxygen consumption was measured using the YSI 5300A Biological Oxygen Monitor, which is a polarographic Clark-style oxygen electrode, as previously described (22).

XF24 Extracellular Flux Analyzer measurements

Cells were seeded (60,000 cells/well) in 24-well plates from Seahorse Biosciences (Billerica, MA). The following day they were treated with drug for 16 hours before measuring their oxygen consumption rate (OCR). One hour prior to the assay, culture medium was replaced with modified DMEM supplemented with 1 mM sodium pyruvate, 1 mM glutamate and 5 mM glucose (pH 7.4). The rate of oxygen consumption (OCR) was measured at 37°C using an XF24 Extracellular Flux Analyzer from Seahorse Bioscience. The baseline (basal) oxygen consumption rate (OCR) was measured three times before and three times after each sequential injection of oligomycin (1 μ M), FCCP (0.8 μ M) and rotenone (both 1 μ M). At the end of the assay, protein concentration was determined for individual wells as described previously (21). To account for variations in cell number

brought about by drug-induced effects on proliferation or cell death, all raw OCR values were normalized to total protein content.

Mouse studies

Pathogen-free female Ncr-*nu/nu* mice were obtained from the National Cancer Institute (stock # 01B74) Taconic Industries (Germantown, NY) and housed in the animal facilities of University Laboratory Animal Resources and the Institute for Human Gene Therapy of the University of Pennsylvania. All experiments were carried out in accordance with University Institutional Animal Care and Use Committee guidelines.

Tissue Oxygen Measurements

The OxyLab pO₂TM single chamber oxygen monitor (Oxford Optronix Ltd., Central 127, Milton Park, OX14 4SA, U.K.) was used to monitor tissue oxygen levels in mice bearing subcutaneous flank tumors. This technique has been described previously (23). Prior to the start of drug treatment baseline oxygen levels were determined for each mouse in the control as well as the BEZ-treated group. Mice were anesthetized with isoflurane before inserting the probe longitudinally through the tumor. The probe was then retracted through the tumor stopping several times to record the pO₂ along the longitudinal axis. An average was calculated from 4–6 readings through one track.

Immunohistochemistry and Fluorescence Microscopy measurements

The hypoxia marker EF3 [(2-(2-nitroimidazol-1 [H]-yl)-N-(3, 3, 3-trifluoropropyl) acetamide)] which forms long-lasting covalent bonds with hypoxic cells was used to label hypoxic regions in tumors. EF3 dissolved in saline (20 mM) was injected into tumor bearing mice 3 hours prior to tumor removal via the tail vein at 0.01 ml/g. Two hours prior to tumor removal a second injection of EF3 (0.03ml/g) was given by intra-peritoneal injection. Hoechst (3mg/ml in saline) was injected (0.01ml/g) 1 minute before tumors were removed.

Cryosectioning, immunohistochemistry, and fluorescence microscopy for EF3 were performed as described previously (13). Briefly, sections (20-um thickness) were cut and they were fixed with 4% PF, rinsed in Dulbecco's PBS (Sigma), and blocked in PBS containing 0.3% Tween 20 and 1.5% albumin, plus 20% nonfat milk and 5% normal mouse serum. Antibody staining for EF3 was performed for 4.5–5 hours using a monoclonal antibody (ELK5-A8) conjugated to the fluorochrome Cy5 (Amersham Life Sciences, Arlington Heights, IL). Slides were rinsed in PBS containing 0.3% Tween 20 and PBS without Tween 20, and then stored in 1% PF. Images were taken on a Zeiss Axio Observer Z1 microscope using Zen 2011 software. A total of 82 tiles were taken for each tumor and the intensity was determined using Image-J software. All images were photographed the same day using the same exposure time.

Construction of point mutated PDH plasmids and infection into cells

pCMV6 plasmid containing Flag-tagged PDHA1 cDNA sequence was purchased from Origene (Rockville, MD). QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) was used to substitute serine with alanine. Briefly, the entire plasmid was amplified using PCR with primers containing the desired mutation using

PfuUltra DNA polymerase. Following amplification, template plasmids were digested with Dpn I and mutated plasmids were used to transform competent cells. Mutations were confirmed by sequencing plasmids. The wildtype and mutant PDHA1 cDNAs were excised and placed into the pBABE-Neo vector.

5×10^5 293T cells were plated per 100 mm plate. Transfections of pBABE plasmids containing wildtype or mutant PDHA1 were performed with Lipofectamine 2000 according to the manufactures protocol (Invitrogen, Grand Island, NY).

SQ20B cells to be infected were seeded 1×10^6 cells per 100 mm dish 24hrs before infection. On the day of infection, virus-containing media was collected and centrifuged to remove floating cells and debris (1000 rpm for 5 min). Polybrene was added to the virus containing media to a final concentration of 8 microgm/ml before placing on the target cells. The 293T cells were fed with fresh media, which was used to infect cells as above a second time. Twenty-four hours following the first day infections the cells were infected a third time and allowed to grow for 48 hr before placing them under selection with G418.

Results

We previously demonstrated that the anti-HIV agent nelfinavir, which happens to inhibit PI3K signaling, can decrease hypoxia in human tumor xenografts (18). Others have shown that more specific drugs such as NVP-BEZ235 (henceforth referred to as BEZ235), which is a dual inhibitor of the p110 subunit of PI3K and mTOR (24) can also decrease hypoxia (25). We set out to understand how this occurred. One potential explanation for this reduced tumor hypoxia is that the tumor cells decreased their consumption of O_2 in response to drug treatment. To test this, we measured O_2 consumption *in vitro* using the YSI 5300A Biological Oxygen Monitor. Since the cells are suspended in a sealed chamber, the decrease in O_2 measure as a function of time is a direct measure of cellular O_2 consumption rate (OCR). We used BEZ235 at a concentration of 50 nM, which decreases phosphorylation of Akt as well as the mTOR targets S6 and 4E-BP1 (Suppl. Fig. 1A). Treatment of SQ20B cells with BEZ235 resulted in a significant decrease (37%) in OCR compared to vehicle only-treated cells (Fig. 1A). Trypan blue exclusion assay of SQ20B cells incubated with BEZ235 for 16 hours showed no significant difference in cell viability compared to vehicle treatment (data not shown), indicating that the decrease in OCR by BEZ235 was not caused by drug-induced cell death. We found no evidence that treatment of these cells with BEZ235 led to apoptosis, as there was no increased in cleaved caspase 3 (Suppl. Fig. 1B), consistent with our published results showing no effect of the drug on PARP cleavage (26).

To further confirm our observation regarding OCR we used the XF Extracellular Flux Analyzer (27), which uses fluorescence quenching to measure dissolved O_2 . Fig. 1B shows the tracings from an experiment in which we started the measurement 16 hours after BEZ235 treatment (corresponding to $T = 0, 8, 16$ minutes). BEZ235 treatment decreased OCR by 44% compared with control cells, similar to that seen using the Clark electrode. To determine whether the decrease of OCR in SQ20B cells was specific for BEZ235, we treated cells with an alternate dual PI3K/mTOR inhibitor BGT226 (28) at a dose that inhibits phosphorylation of Akt, S6 and 4E-BP1 (50 nM) (Suppl. Fig. 1C). Treatment with BGT226

showed a similar decrease in basal OCR which was comparable to that observed following treatment with BEZ235 (Fig. 1B) Thus taken together these results indicate that pharmacological inhibition of PI3K/mTOR pathway decreases basal OCR.

Both electron transport chain (ETC) activity and the rate of ATP production in the mitochondria determine the OCR. These two processes are coupled via the proton gradient in normally functioning cells. To assess the specific contributions of ATP synthesis and ETC activity to altered OCR, we performed measurements in real-time following sequential addition of oligomycin (an ATP synthase inhibitor) and FCCP (an ionophore that dissipates the proton gradient thereby uncoupling proton pumping from ATP synthesis) (Fig. 1B). As expected, following the addition of oligomycin (line A) OCR was decreased in all treatment groups, although it did not go to zero (likely due to proton leak). After the addition of FCCP (line B) there was a robust increase in OCR in the control group (OCR at T = 48, 56 64 minutes), which represents the maximal mitochondrial O₂ consumption. However, treatment with BEZ235 or BGT226 reduced uncoupled respiration compared to controls. Thus, both coupled and uncoupled respirations are inhibited by PI3K/mTOR inhibition. As expected, addition of the irreversible complex I inhibitor rotenone (line C) led to inhibition of O₂ consumption in control and drug-treated groups.

To determine the kinetics at which BEZ235 or BGT226 exert their inhibitory effect on O₂ consumption, cells were incubated in either drug for varying lengths of time (1–16 hours) before measuring OCR in the flux analyzer. Treatment of either drug for 1 or 2 hrs prior to measurement had little effect on OCR. However, a statistically significant reduction in OCR was observed after 4, 8 and 16 hours of BEZ235 treatment (Suppl. Fig. 2).

Both BEZ23 and BGT226 inhibit PI3K and mTOR; however, we have not ruled out the possibility that their effect on OCR may be the result of some off-target effect. To test this, we used siRNA to knock down Akt1, which is directly downstream of PI3K. As shown in Fig. 1C, inhibition of Akt1 expression led to a corresponding decrease in P-Akt and a transient decrease in P-S6 (at 48 hrs.). Using the flux analyzer we measured OCR in cells transfected with Akt1 siRNA or control siRNA. The baseline OCR readings (at T = 0 hours) as shown in Fig. 1D indicates that knockdown of Akt1 resulted in a GEORGE: 37% decrease in OCR. We also used KU-0063794, which is a specific inhibitor of mTOR (29), leading to decreased phosphorylation of both P-S6 and P-4EBP1 (Fig. 1E). Treatment of cells for 16 hrs with this drug did not reduce OCR (Fig. 1F).

To further determine the mechanism by which these drugs decreased O₂ consumption, we investigated the possibility of some structural or functional change induced in the mitochondria. Using Mito-Tracker Green (Invitrogen M7514), which localizes to mitochondria regardless of membrane potential, we found no difference in signal intensity between the BEZ235-treated and vehicle only-treated groups (Suppl. Fig. 3A); hence, drug treatment did not substantially alter mitochondrial mass. We also examined mitochondrial membrane potential (MMP) using Mito-Tracker Red (Invitrogen M22425), a stain that accumulates in live cells in a manner dependent upon MMP, and found no difference in signal intensity between the BEZ235-treated and vehicle only-treated groups (Suppl. Fig. 3B). Finally, analysis of the DNA levels by primers specific to the mitochondrial gene

COX1 by RT-PCR (Suppl. Fig. 4, Table S1) did not change significantly in response to BEZ235 treatment.

We subsequently examined whether drug treatment might alter mitochondrial respiratory chain activity. A major regulator of mitochondrial oxidative function is the pyruvate dehydrogenase complex (PDC) which catalyzes the irreversible decarboxylation of pyruvate to form acetyl-coA, which can then enter the citric acid cycle and be used as a substrate for oxidative phosphorylation (30). PDC activity is inhibited by phosphorylation of its pyruvate dehydrogenase (PDH) E1 α subunit (30). Hence, phosphorylation of PDH-E1 α reduces entry of pyruvate into the citric acid cycle and consequently decreases O₂ consumption. We found that incubation of SQ20B cells with either BEZ235 or BGT226 led to a time-dependent increase in of PDH-E1 α phosphorylation, which was detectable within 2 hours and continued to increase for up to 16 hours (Fig. 2A). No change in the level of PDH-E1 α , E1 β , E2, or E2/E3bp was seen (Fig. 2B, Suppl. Fig. 5). For the remainder of this manuscript for simplicity's sake we will refer to PDH-E1 α , as simply PDH.

To test whether inhibition of the PI3K/Akt pathway was necessary for the drug-induced effect on PDH phosphorylation, we used siRNA to knock down Akt1. Inhibition of Akt1 resulted in a 4.5-fold increase in Ser293 phosphorylation of PDH (Fig. 2B), similar to that seen with either BEZ235 or BGT226. In this particular experiment, in which samples were harvested at 72 hours following transfection, Akt1 siRNA had no effect on decreasing S6 phosphorylation (which is directly downstream of mTOR). However, this still resulted in increased PDH-E1 α phosphorylation, suggesting that Akt itself rather than mTOR might be responsible for this effect. As a complementary approach, we used the drug GDC-0068, an Akt inhibitor currently being tested in clinical trials (31), and GDC-0980, another dual PI3K/mTOR inhibitor (32). Incubation with GDC-0068 actually increased Akt phosphorylation as has been reported previously for this drug and other ATP-competitive Akt inhibitors (31). However, as expected, there was decreased phosphorylation of the downstream targets S6 and 4E-BP1 (Fig. 2C). There was a concomitant increase in PDH-E1 α phosphorylation with GDC-0068 treatment, similar to what we observed with BEZ235, BGT226, and Akt1 siRNA. Flux analyzer measurements showed that the OCR was also reduced with GDC-0068 treatment by 64% (Fig. 2D and Suppl. Fig. 6A). Treatment with GDC-0980 showed similar effects on PDH-E1 α phosphorylation and OCR (Fig. 2C and 2D, Suppl. Fig. 6B). Lastly, we used MK-2206, which, unlike GDC-0068, is a non-ATP competitive allosteric Akt inhibitor (33). In contrast to GDC-0068, MK-2206 does decrease Akt phosphorylation (Fig. 2E). It also led to decreased OCR (Fig. 2F)

To generalize these results we used another head and neck cancer cell line, FaDu. There was a time-dependent increase in PDH phosphorylation between 2 and 16 hours of exposure to either BEZ235 or BGT226 (Fig. 3A). Using the flux analyzer we found that 8 hours of incubation with BEZ235 or BGT226 resulted in significant decreases in OCR (Fig. 3B and Suppl. Fig. 6C).

To further test the relationship between Akt and PDH phosphorylation using a genetic approach, we used U251 glioblastoma cells engineered to express wildtype PTEN under the control of a tetracycline-inducible promoter (34). These cells express constitutively high

levels of P-Akt due to their mutant PTEN status. Addition of doxycycline caused a substantial decrease in P-Akt, and an increase in PDH phosphorylation (Fig. 3C). Importantly, this did not occur in a control cell line that expresses a mutant form of PTEN that cannot decrease Akt phosphorylation, indicating that this was not simply a response to doxycycline (Fig. 3C). We also found that addition of doxycycline led to a 46% decrease in the basal OCR in the cells induced to express wildtype PTEN (Suppl. Fig. 7A and Fig. 3D), further supporting the link between Akt and O₂ consumption. Notably, this decrease in the basal OCR did not occur when mutant PTEN was induced in U251-C124S cells (Suppl. Fig. 7B). We determined whether PDH was phosphorylated in this cell line and found Ser293 phosphorylation occurred with similar kinetics as noted in SQ20B and FaDu cells (Fig. 3E). We also found that PDH was phosphorylated at both Ser232 and Ser300 in response to treatment with either drug.

To test the importance of PDH on O₂ consumption in SQ20B cells, we knocked down the PDH-E1 α subunit using siRNA (Fig. 4A; compare lanes 4 and 2). Reduced PDH resulted in a 32% decrease in basal O₂ consumption (Fig. 4B and Suppl. Fig. 8A). Treatment of cells with BEZ235 or BGT226 led to a similar decrease in OCR.

As a means of examining the importance of PDH-E1 α phosphorylation in the ability of BEZ235 to regulate O₂ consumption, we treated cells with DCA (dichloroacetate). DCA treatment decreased Ser293 phosphorylation (Fig. 4C; compare lanes 1 and 2), through inhibition of pyruvate dehydrogenase kinases (PDKs) as previously reported (35). Treatment of cells with DCA blunted the increase in PDH phosphorylation seen in response to BEZ235 (Fig 4C: lane 3 vs. 4) or to BGT226 (lane 5 vs. 6). Treatment of cells with DCA along with BEZ235 reversed the effect of BEZ235 on decreasing OCR (Fig. 4D) and Suppl. Fig. 8B). These figures also show that DCA had a similar abrogating effect on the decrease in OCR seen with BGT226 treatment.

We also used a genetic means to establish that the phosphorylation of PDH E1 α was essential for the ability of BEZ235 to decrease O₂ consumption. We infected SQ20B cells with retrovirus expressing FLAG-tagged wildtype PDH-E1 α or PDH-E1 α containing a single S1 (S232A) or triple S3 (S232A, S292A, S300A) serine to alanine mutation(s) (Fig. 5A). Fig. 5B shows that an anti-FLAG antibody recognized a FLAG-tagged protein in the cells infected with a PDH-E1 α (wildtype or mutant) virus (lanes 2–4). Using an antibody recognizing P-PDH (S232), we found that in wildtype PDH-E1 α -infected cells there were two separate proteins (marked by the two arrows, second row). The bottom band corresponds to the endogenous protein and upper band the FLAG-tagged exogenous protein. This upper band is not visible in lanes 3 and 4 because these cells express mutant PDH-E1 α in which the S232 has been altered to alanine, hence the exogenous protein is not recognized by this antibody. Suppl. Fig. 9 shows another immunoblot of lysates from cells infected with the 1S or 3S PDH mutant probed using 3 different antibodies. The P-PDH (S232) antibody recognizes both the endogenous and the exogenous proteins. However, the P-PDH (S293) and P-PDH(S300) antibodies appear to only express the exogenous protein. Of note, the 3S mutant does suppress phosphorylation of the endogenous PDH at S293 as well as S300 (compare lane 10 with 8 or 6).

We treated these cells with BEZ235, then performed OCR measurements. Fig. 5C shows that the cells expressing FLAG-tagged wildtype PDH-E1 α exhibited a similar decrease in OCR in response to BEZ235 treatment as did control cells. This is more readily appreciated in Fig. 5D in which the same data are plotted to show the % decrease in OCR in response to BEZ235. The % decrease in OCR in cells expressing wildtype PDH-E1 α was not different than in control cells ($p = ns$). However, the % decrease in OCR in cells expressing either the single (or triple) mutant PDH was significantly different than in control cells ($p = 0.004$). Hence, expression of mutant PDH-E1 α that cannot be phosphorylated on Ser232 blunts the effect of BEZ235 on decreasing OCR.

SQ20B human squamous head and neck cells were grown as tumor xenografts in nude mice and were then injected with EF3, a nitroimidazole that forms adducts with proteins in hypoxic regions (36). Treatment of mice with 50 nM BEZ235 led to a significant decrease in EF3 binding (Figs. 6A, B) when compared to vector only treated tumors ($p = 0.05$), indicating a decrease in overall tumor hypoxic fraction. As an alternate method of assessing the effect of BEZ235 within tumor xenografts, we used the OxyLabTM pO₂ probe. SQ20B xenografts were grown in nude mice. Drug treatment was not started until tumors were at least 400 mm³ in size (400 to 1800 mm³). pO₂ measurements were made prior to start of drug (day 0), at day 1 and at day 3. Each tumor served as its internal control, so the pO₂ was compared to the Day 0 readings for that particular tumor. We calculated the fold change in pO₂ at Day 1 or Day 3 relative to the Day 0 reading (Fig. 6D). In BEZ235-treated mice, there was nearly a five-fold increase in pO₂ both at day 1 and at day 3 relative to day 0. We also had a set of control tumors in mice not treated drug. In these tumors, there was no appreciable change in pO₂ at day 1 or day 3 relative to day 0 (Fig. 6D). The fold-difference in pO₂ in BEZ-235 treated tumors was statistically significant compared to the fold-difference seen in control (non drug-treated) mice at both Day 1 ($p = 0.026$) and Day 3 ($p = 0.007$). The mean pO₂ for control (non-drug treated) tumors was 0.95 mm Hg at day 1 and 0.65 mm Hg at day 3. For BEZ235-treated tumors it was 2.1 and 3.0 mm Hg respectively.

Following pO₂ measurement on day 3, mice were sacrificed and tumors were removed to measure the *in vivo* level of PDH 293 phosphorylation by immunoblot analysis (Fig. 6E). Most BEZ-235 treated tumors showed a decrease in P-Akt and an increase in P-PDH (S293). The mean level of phosphorylation BEZ-treated mice was 2.08 compared to 1.04 for control, non-drug treated mice. This difference was found to be significantly different ($p = 0.009$).

Discussion

The presence of hypoxia within human tumors has been associated with resistance to therapy. For decades this has been appreciated in the case of radiotherapy due to the fact that O₂ must be present for optimal fixation of DNA damage induced by ionizing radiation (37). There is particularly strong evidence in head and neck cancers that hypoxia plays an important role in resistance to radiotherapy (38–40). There are also reports indicating that hypoxic cells are more resistant to killing following exposure to commonly used cytotoxic agents such as cisplatin, etoposide and doxorubicin (9–11, 13). Tumor hypoxia has typically been attributed to impaired blood flow due to the disorganized vasculature often found in

tumors (16). However, our results suggest that genetic mutations that activate the PI3K/mTOR pathway may lead to increased hypoxia by increasing tumor oxygen consumption.

There are currently numerous ongoing clinical trials PI3K inhibitors in patients with cancer. In the current study we show that treatment of mice bearing tumor xenografts with the dual PI3K/mTOR inhibitor BEZ235 decreased *in vivo* tumor hypoxia. To demonstrate this we used both the nitroimidazole EF3 and OxyLab pO₂ probe measurements. We measured the effects of two different PI3K/mTOR inhibitors and two different Akt inhibitors on *in vitro* oxygen consumption using the Clark electrode and/or the flux analyzer. As these drugs could have off-target effects, we used genetic approaches including RNAi and cells with inducible PTEN to confirm that it was indeed inhibition of this pathway that was specifically responsible for the decrease in oxygen consumption. Similar findings on tumor O₂ consumption have been made by others using PI3K inhibitors, although these studies did not report any potential mechanism and were focused on pharmacologic inhibition of the pathway (41). However, in further investigating potential molecular mechanisms underlying this effect, we found that PI3K/Akt/mTOR inhibition led to increased PDH-E1 α phosphorylation (i.e decreased activity of the enzyme). As PDH is the critical determinant as to whether pyruvate is converted to acetylCoA, which can then participate in the tricarboxylic acid cycle, inhibiting activity of this enzyme should reduce O₂ consumption. Hence PDH phosphorylation offers an explanation as to how PI3K/mTOR inhibition can decrease O₂ consumption and reduce tumor hypoxia. siRNA directed against PDH-E1 α decreased OCR, similar in extent to that seen with either BEZ235 or BGT226 (Fig. 4B). Dichloroacetate treatment, which prevents the PDH phosphorylation in response to PI3K/mTOR inhibition, also reversed the effect on OCR (Fig. 4D). Ectopic expression of PDH-E1 α that was mutated so that the serine sites could not be phosphorylated resulted in a blunting of the ability of BEZ235 to decrease OCR. Hence, our evidence supports a causal relationship between PDH-E1 α phosphorylation in response to PI3K/mTOR inhibition and reduced O₂ consumption.

We started our studies using BEZ235, which inhibits both PI3K and mTOR; however, we also we found that Akt1 siRNA increased PDH-E1 α phosphorylation. This occurred in spite to the fact that S6 phosphorylation was not affected (Fig. 2B), which leads us to believe that it is Akt rather than mTOR that regulates this phosphorylation. Our results do not preclude the possibility that the PI3K/mTOR pathway may regulate other molecular changes that could contribute to mitochondrial respiration. mTOR itself it has been implicated in the regulation of oxygen consumption (42–44). Interestingly, mTOR has been shown to regulate the translation of certain mitochondria-related mRNAs (45). However, our results uncover a novel link between the PI3K/Akt pathway and PDH phosphorylation which play an essential role in the regulation of O₂ consumption. PDH is one of the key players in the regulation of oxidative metabolism. Papandreou *et al.* showed that PDH phosphorylation is increased under hypoxia (46), and PDH has recently been implicated in cellular senescence (47).

An unanswered question is why the PI3K/Akt pathway should result in greater oxidative metabolism. During oncogenic transformation this pathway is co-opted; however its original function is in normal growth and development. This pathway is activated by growth factor receptor signaling and appears to be important during proliferation, which is an energy

requiring process. Hence, increased oxidative phosphorylation could play a teleologic role as a means of generating more ATP from each molecule of glucose.

In addition to identifying a new mechanism by which the PI3K/Akt pathway regulates metabolism, our results also have potential clinical implications. As mentioned previously, the presence of hypoxia is a negative prognostic factor in cancers treated definitively with radiation including head and neck Ca (38, 40) and prostate Ca (48). A recent review and meta-analysis of clinical trials using manipulations that target the hypoxic fraction in head and neck SCC concluded that hypoxic modification of radiotherapy led to increased loco-regional control, disease-specific survival and overall survival (14, 15). Another randomized trial showed that the addition of carbogen breathing and nicotinamide to decrease tumor hypoxia improved outcome in patients with laryngeal cancer treated with radiation (49). However, in spite of these suggestive results, these manipulations that aim to address the problem by increasing the supply of oxygen have shown marginal benefit and have not gained widespread adoption.

An alternative strategy to reduce tumor hypoxia is to attack the problem on the demand side, i/e reducing cancer cell O₂ consumption. By decreasing the O₂ consumption of tumors, there should be more O₂ available to better oxygenate the tumor both intracellularly and extracellularly, which is what we have found. In fact, mathematical modeling from Secomb *et al.* showed that reducing O₂ consumption rate may be more effective than elevating blood flow or oxygen content as a method to reduce tumor hypoxia. (50). These authors found that hypoxia (< 3 mm Hg) was abolished by a reduction in consumption rate of at least 30%. Recently the diabetes medication metformin was reported to inhibit O₂ consumption, and the authors proposed that it might be effective in combination with radiotherapy by reducing tumor hypoxia (51). Our findings indicate that PI3K inhibitors currently in clinical trials may also be useful in this regard. In future trials, this hypothesis could be tested by using non-invasive hypoxia imaging. Such imaging is available with a number of agents including ¹⁸F-misonidazole, ¹⁸F-EF5, and ¹⁸F-IAZA (reviewed in (52)).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. PI3K/mTOR inhibition reduces O₂ consumption of SQ20B cells *in vitro*

(A) After 16 hrs. of treatment with BEZ235 (50 nM), SQ20B cells were harvested as single cells and placed in Clark electrode chambers for O₂ measurement. Slopes of lines represent rate of O₂ consumption. Bar graph to right of line graph shows O₂ consumption rate (OCR) determined from these slopes (*: $p = 0.014$). (B) Cells were seeded into flux analyzer plates and allowed to attach before BEZ235 or BGT226 was added for 16 hours prior to measurement of OCR. Vertical lines labeled A, B, and C indicate respectively times when ATPase inhibitor oligomycin, mitochondrial uncoupler FCCP, or complex I inhibitor rotenone was added. Bar graph to right of flux analyzer tracing shows baseline OCR as determined from flux analyzer tracing at start of measurements (average of readings at 0, 8, and 16 minutes). (*: $p = 0.001$; **: $p = 0.006$). (C) SQ20B cells were transfected with scrambled siRNA or siRNA directed against Akt1. 48, 72 or 96 hours later cells were trypsinized and Western blotting was performed. Numbers below P-Akt lane represent fold-increase in intensity relative to lane 1. (D) cells were plated into flux analyzer plates and allowed to attach prior to OCR measurement. This panel shows bar graph using T=0 measurements from flux analyzer tracing (*: $p = 0.006$). (E) Cells were treated with KU-0063794 for indicated lengths of time prior to harvesting. Lysates were collected, and immunoblotting was performed. (F) Cells were seeded into flux analyzer plates and allowed to attach before KU-0063794 was added for 16 hours prior to measurement of OCR. Bar graph shows baseline OCR as determined from flux analyzer tracing (at start of measurements).

Figure 2. PI3K/Akt/mTOR inhibition increases E1 α phosphorylation in SQ20B cells

Panels (A) – (C) are immunoblots using antibodies as indicated. Fold increase refers to increase in P-PDH-E1 α (Ser293) relative to baseline lane, which is given the value 1.0. (A) SQ20B cells were treated with BEZ235 or BGT226 for indicated lengths of time prior to harvesting. Numbers below P-PDH (S293) lane represent fold-increase in intensity relative to lane 2. (B) Cells were harvested 48 hours after transfection with Akt1 siRNA or 16 hours after BEZ235 or BGT226 (50 nM) treatment. Numbers below P-PDH (S293) lane represent fold-increase in intensity relative to lane 1. (C) SQ20B cells were treated with GDC-0068 (5 μ M) or GDC-0980 (1 μ M) for indicated lengths of time before harvest. Numbers below P-PDH (S293) lane represent fold-increase in intensity relative to lane 1. (D) SQ20B cells were seeded into flux analyzer plates and allowed to attach before GDC-0068 or GDC-0980 was added for 16 hours prior to measurement of OCR. Bar graph (2D) shows baseline OCR as determined from flux analyzer tracing (Suppl. Fig. 6A, B) at start of measurements (average of readings at 0, 11, and 21 minutes). (*: p = 0.004). (E) Same as in (D) except MK-2206 was added for 16 hours prior to measurement of OCR. Lysates were collected, and immunoblotting was performed. (F) Cells were seeded into flux analyzer plates and allowed to attach before MK-2206 was added for 16 hours prior to measurement of OCR. Bar graph shows baseline OCR as determined from flux analyzer tracing (at start of measurements).

Figure 3. PI3K/mTOR inhibition decreases O₂ consumption and increases PDH-E1α phosphorylation in other cell lines

(A) FaDu cells were treated with BEZ235 or BGT226 for indicated lengths of time prior to harvesting. Lysates were collected, and immunoblotting was performed. Fold increase refers to increase in P-PDH-E1α (Ser293) relative to baseline lane, which is given the value 1.0. (B) FaDu cells were seeded into flux analyzer plates and allowed to attach before BEZ235 or BGT226 was added for 16 hours prior to measurement of OCR. Bar graph shows baseline OCR as determined from flux analyzer tracing (Suppl. Fig. 6) at start of measurements (average of readings at 0, 11, and 21 minutes). (*: $p = 0.004$; **: $p = 0.008$). (C) U251MG cells engineered to be inducible for either wild-type PTEN or phosphatase-dead PTEN (C124S) were exposed to doxycycline (1μg/ml). After 24 hours, cells from each group were harvested and the resultant lysates immunoblotted. (D) U251-PTEN cells were treated with doxycycline as described in panel C, then placed into flux analyzer plates for OCR measurement. Flux analyzer tracing is shown in Suppl. Fig. 7A. Lines A, B, C represent times when oligomycin, FCCP, and rotenone were added respectively. Bar graph (Fig. 3D) representing baseline OCR taken from flux analyzer tracing. (*: $p = 0.0001$). (E) U251-wtPTEN cells were treated with BEZ235 (50 nM) or BGT226 (50 nM) for indicated lengths of time prior to harvesting. Lysates were collected, and immunoblotting was performed. Fold increase refers to increase in P-PDH-E1α (Ser293) relative to baseline lane, which is given the value 1.0.

Figure 4. Downregulating PDH decreases O₂ consumption and reversing drug-induced PDH phosphorylation abrogates effect of PI3K inhibitors on O₂ consumption

(A, B) SQ20B cells were seeded and allowed to attach prior to transfection with siRNA PDH-E1α (50 nM). Parallel dishes of cells were treated with BEZ235 (50 nM) or BGT226 (50 nM). Either 48 hours after siRNA transfection or 16 hours after drug treatment, (A) cells were harvested and immunoblotting was performed or (B) OCR measurements were made on the flux analyzer. Bar graph shows baseline OCR as determined from flux analyzer tracing (Suppl. Fig. 8A) at start of measurements (average of readings at 0, 11, and 21 minutes). The difference between the scrambled siRNA and any of the other 3 groups, PDH siRNA, BEZ235 or BGT266 was statistically significant (*: p = 0.00001). (C, D) Cells were treated with 20 mM DCA (dichloroacetate) for one hour prior to being treated for 16 hours with either BEZ235 or BGT226. Either (C) immunoblotting was performed and numbers below P-PDH (S293) lane represent fold-increase in intensity relative to lane 1. (D) OCR measurements were made on the flux analyzer. Bar graph shows baseline OCR as determined from flux analyzer tracing (Suppl. Fig. 8B) at start of measurements (average of readings at 0, 11, and 21 minutes). (*: p = 0.0002, **: p = 0.00003).

Figure 5. Ectopic expression of PDH-E1α mutants that cannot be phosphorylated blunts BEZ235-mediated decrease in OCR

(A) Schematic representation of plasmids encoding C terminal FLAG-tagged mouse PDHA1 (WT E1α) and single point mutation of serine232 -> alanine at position (1S-1A) or triple mutation of serine232, serine 292, and serine 300 -> alanine (3S-3A). (B) SQ20B cells were infected with retrovirus expressing plasmids encoding WT E1α or 1S-1A mutant or 3S-3A mutant. 72 hours later cells were trypsinized and Western blot analysis was performed for indicated proteins. (C) Alternatively similarly infected cells were plated into flux analyzer plates and allowed to attach prior to OCR measurement. The bar graph shows the OCR values for control and infected cells +/- BEZ235 treatment. (D) The same OCR data from (C) are presented as % change (decrease in OCR following BEZ235 treatment divided by OCR without BEZ235 treatment). (*: $p = 0.004$; **: $p < 0.001$).

Figure 6. BEZ235 decreases tumor hypoxia in human xenografts

(A, B) When the mice were 5 to 7 weeks of age, tumors were initiated in the flank by subcutaneous injection of 1×10^6 SQ20B cells. Starting 10–14 days later, when tumors were 50–100 mm³ in size, mice in the treatment group were gavaged daily with BEZ235 (50 mg/kg) suspended in 0.1% Tween 80 and 0.3% carboxymethylcellulose in sterile physiological saline. After 5 days of drug treatment, the mice were injected with EF3. 1.5 hours later they were sacrificed, and the tumors were removed and stained using an antibody to EF3. Panel A shows representative samples with staining. Panel B shows the quantitation of EF3 binding as bar graph (*: $p = 0.05$). (C) SQ20B xenografts were grown subcutaneously in nude mice. Tumors were measured every two days through the experiment and plotted in panel. (D) When tumors reached a size of at least 400 mm³, mice were assigned to either control (4 tumors) or BEZ235 treatment (6 tumors). The OxyLab pO₂TM probe was used to measure pO₂ levels (see Materials and Methods) for the control and BEZ-treated tumors on day 0 (just prior to drug treatment) and then on day 1 and day 3 following the start of drug treatment. Each animal served as its own control, and the fold change was calculated relative to the day 0 measurement for each individual mouse and plotted in whisker graph. (E) Following pO₂ measurement on day 3, mice used in (D) were sacrificed and tumors were removed to measure the *in vivo* level of PDH 293 phosphorylation by immunoblot analysis. Numbers below P-PDH (S293) lane represent fold-increase in intensity relative to lane 1.