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Original article

Regulation of O_2 consumption by the PI3K and mTOR pathways contributes to tumor hypoxia

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ABSTRACT

Background: Inhibitors of the phosphatidylinositol 3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) pathway are currently in clinical trials. In addition to antiproliferative and proapoptotic effects, these agents also diminish tumor hypoxia. Since hypoxia is a major cause of resistance to radiotherapy, we sought to understand how it is regulated by PI3K/mTOR inhibition.
Methods: Whole cell, mitochondrial, coupled and uncoupled oxygen consumption were measured in cancer cells after inhibition of PI3K (Class I) and mTOR by pharmacological means or by RNAi. Mitochondrial composition was assessed by immunoblotting. Hypoxia was measured in spheroids, in tumor xenografts and predicted with mathematical modeling.
Results: Inhibition of PI3K and mTOR reduced oxygen consumption by cancer cell lines is predominantly due to reduction of mitochondrial respiration coupled to ATP production. Hypoxia in tumor spheroids was reduced, but returned after removal of the drug. Murine tumors had increased oxygenation even in the absence of average perfusion changes or tumor necrosis.
Conclusions: Targeting the PI3K/mTOR pathway substantially reduces mitochondrial oxygen consumption thereby reducing tumor hypoxia. These alterations in tumor hypoxia should be considered in the design of clinical trials using PI3K/mTOR inhibitors, particularly in conjunction with radiotherapy.
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Genetic alterations that lead to aberrant activation of the PI3K and mTOR pathways are among the most frequent in cancers of all types [8,40]. PI3K/mTOR pathway signaling promotes a plethora of oncogenic cellular functions including growth and cell survival. As a result, a variety of pharmacologic agents have been developed to inhibit this pathway and are entering clinical trials [25].

In addition to reducing tumor proliferation and survival, we and others have shown that inhibitors of the PI3K pathway greatly diminish tumor hypoxia [14,38,53], a characteristic feature of solid tumors which correlates with poorer prognosis, increased metastasis and resistance to therapeutic intervention, particularly radiotherapy (XRT) [18,33,34]. A variety of strategies to increase tumor oxygenation have been proposed in order to improve the outcome of XRT, including the inhalation of hyperoxic gases [20–23,42], and vascular normalization [10,19,41]. Poor local perfusion is a major contributor to hypoxia, resulting from the disorganized vasculature, increased interstitial pressures and transient vascular closures commonly found in cancer [50]. Inhibition of PI3K signaling results in improved vascular structure and perfusion [14,38], suggesting that vascular normalization plays a role in the observed reoxygenation. However, potential contributions of alterations in oxygen consumption have not been determined. Mathematical modeling of the roles of supply and demand in tumor oxygenation suggests that reduction of hypoxia is more likely to be accomplished by decreasing tumor oxygen consumption than by improvement of vascular supply [45].

Consistent with this idea, Gallez and colleagues have shown that treatment of some murine tumors with inhibitors of the Epidermal Growth Factor Receptor (EGFR) or with AsO_3 leads to decreased O_2 consumption and hence decreased hypoxia in experimental tumors correlating with enhanced efficacy of XRT [3,12]. The effects of inhibiting PI3K and mTOR on O_2 consumption and tissue oxygenation however, have not been similarly explored.

Here we report that pharmacological and genetic inhibition of PI3K and/or mTOR leads to diminished O_2 consumption in a panel of cell lines from different primary tumors. Using spheroid and

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in vivo models, we found that tissue hypoxia was reduced even in the absence of vascular remodeling by pharmacological inhibition of PI3K/mTOR.

Methods

In vitro studies

The cell lines tested FaDu (human nasopharyngeal), EMT6 (mouse mammary carcinoma), HCT116 (human colorectal) and U87 (human glioma) were chosen because they form spheroids of sufficient size (~400–500 μm diameter) to become hypoxic. Spheroids generated using the liquid overlay technique [9] were treated 6 days after initiation.

Treatments

BEZ235 and BKM120 (Novartis Pharmaceuticals) and AZD8055 (#CT-A8055, ChemieTek) were dissolved in 0.1% DMSO. Control groups were treated with equal amounts of DMSO.

Cell transfections were performed by reverse transfection with Interferin (PolyPlus) using 5 nM short interfering RNA (siRNA) oligonucleotides for PI3K110α (#L-003018), p85β (#L-003021), mTOR (#L-003008) and non-targeting (#D-001810) from Dharmacon RNAi Technologies. Doses in tissue culture were chosen based on the lowest concentration to reduce pAKT repeatably and by at least approximately 3-fold.

Immunoblotting

Antibodies were: pAKT (Ser-573 #9271, 1:1000), pmTOR (Ser-2448 #2971, 1:500), pS6 ribosomal protein (Ser235/236 #4856, 1:1000), all Cell Signalling Technology in 5% Bovine Serum Albumin (BSA); Actin (mAb clone AC-15 #A1978, Sigma, 1:20,000 in 5% milk); OXPHOS complexes (#MS601, Mitosciences, 1:500 in milk); citrate synthase (#CISY-11A, Alpha Diagnostic, 1:500 in milk).

Oxygen consumption

Cells were seeded in 96-well plates from Seahorse Biosciences prior to treatment with inhibitors (24 h) or siRNA (48 h). One hour prior to the assay, culture medium was replaced with modified DMEM (Seahorse Bioscience) supplemented with 5 mM sodium pyruvate, 4 mM glutamine, (pH 7.4). The rate of oxygen consumption (OCR) was measured at 37 °C using an XF96 extracellular analyzer (Seahorse Bioscience). Four measurements were taken before and three after each sequential injection of oligomycin (1 μM), FCCP (0.8 μM) and rotenone and antimycin A (both 1 μM). Plates were fixed with 4% paraformaldehyde (pFA) and stained with Hoechst 33342 (H3570, Invitrogen). Cell number was proportional to fluorescence (Ex 380 nM and Em 450 nM). The OCR linked to coupled respiration was obtained by subtracting OCR after the addition of oligomycin from basal OCR. OCR after addition of the mitochondrial uncoupler FCCP reflected the maximal respiratory rate. Non-mitochondrial respiration was defined as the rate after rotenone/antimycin A application and was subtracted from the basal OCR to determine the mitochondrial OCR. "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 cell density measurements as determined by Hoechst fluorescence, on a well-by-well basis. At the end of the assay, plates were fixed with 4% paraformaldehyde (pFA), and stained with Hoechst 33342 (2 μg/ml, H3570, Invitrogen) prior to measuring fluorescence (Ex 380 nM and Em 450 nM). The effect of treatment on cell density with BEZ235 or BKM120 varied across cell lines (Supplemental Fig. 1A).

Isolation of mitochondrial-enriched fraction

Mitochondria were isolated as previously described [16,51]. 2×10^7 cells were centrifuged at 750*g* for 5 min at 4 °C, resuspended in cold PBS, centrifuged and resuspended in isolation buffer (containing 10 mM Tris–HCl, 0.32 M sucrose, 0.1 mM EGTA, and 1:100 Halt Protease Inhibitor Cocktail. After 10 freeze/thaw cycles (−70 °C for 3 m and 37 °C for 2 min), the homogenates were centrifuged at 700*g* for 10 min to remove the nuclei and unbroken cells. Supernatants were centrifuged at 12,000*g* for 10 min to separate the mitochondria and cytosol.

Spheroids

To quantify hypoxia, spheroids were incubated with 200 μM EF5 (2-(2-Nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide), a gift from Cameron Koch's lab) for 6 h at 37 °C, prior to overnight fixation in 4% pFA in PBS and preservation in 30% sucrose in PBS at 4 °C. 8 μm sections were incubated overnight at 4 °C with the EF5 Cy3-conjugated monoclonal antibody ELK3-51 (www.hypoxia-imaging.org) and counterstained with Hoechst 33342. Images were acquired with a Nikon Eclipse 90i with a Hamamatsu ORCA-ER camera. A mask was created for each spheroid using the Hoechst image, prior to measuring the average pixel intensity per spheroid in the background-subtracted EF5 image using ImageJ [1].

To assess signaling inhibition in spheroids, sections were stained with anti-pAKT antibody using ImmPRESS™ reagent kit (MP-7401, VectorLabs) and DAB Peroxidase substrate kit (SK-4100, VectorLabs). Images acquired with a Nikon Eclipse E800 were analyzed for 3,3′-Diaminobenzidine (DAB)-positive pixels semi-automatically using a method described previously [6].

Statistical analysis

Quantitative data were expressed as means ± 95% Confidence Interval. One-way ANOVAs, followed by the Bonferroni post-test, were used to assess the differences among various treatments with Prism 5 (GraphPad Software Inc.), unless otherwise stated.

Mathematical modeling

For simulation of *in vivo* O_2 consumption, 2D vascular networks were generated stochastically [37], using vessel parameter distributions (vessel length, diameter and branching angle) obtained from ex vivo data [14]. The resulting coupled blood flow and O_2 transport problem was solved using code available via Dr. Secomb's website (www.physiology.arizona.edu/people/secomb/greens). A representative base O_2 consumption rate of 1.5 cm^3/100 cm^3/min was used [46].

In vivo studies

All animal experiments were carried out in accordance with UK Home Office regulations. FaDu cells bearing a HRE-luc reporter (Cignal Lenti HIF Reporter luciferase (SABiosciences)), for hypoxia were described and validated previously [38]. 1×10^6 cells were inoculated subcutaneously in athymic nude mice. Mice were divided into cohorts of $n = 4$ mice per cohort. Drug administration by oral gavage was initiated once tumors reached 100 mm^3 volume, as determined using calipers. Tumors were treated for 7 days with BEZ235 at 0, 20 or 30 mg/kg in NMP/PEG300 (Novartis Pharmaceuticals, Basel, Switzerland). Decreases in hypoxia were seen at 3 days with maximal effects by 5–7 days. We failed to observe effects at earlier times. Thus we used 7 days for these experiments. Imaging of hypoxia and perfusion was carried out as previously described [38]. Before sacrifice EF5 was administered as previously described [38]. Tumor tissue was fixed and stained for EF5 and Ki-67. Images were acquired using a Nikon Eclipse E800 microscope with a Nikon DMX1200 digital camera and measured with ImageJ.

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