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PI3K/AKT Signaling Regulates Bioenergetics in Immortalized Hepatocytes

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Abstract

Regulation of cellular bioenergetics by PI3K/AKT signaling was examined in *isogenic* hepatocyte cell lines lacking the major inhibitor of PI3K/AKT signaling, PTEN (phosphatase and tensin homolog deleted on Chromosome 10). PI3K/AKT signaling was manipulated using the activator (IGF-1) and the inhibitor (LY 294002) of the PI3K/AKT pathway. Activation of PI3K/AKT signaling resulted in an enhanced anaerobic glycolysis and mitochondrial respiration. AKT, when phosphorylated and activated, translocated to mitochondria and localized within the membrane structure of mitochondria, where it phosphorylated a number of mitochondrial residence proteins including the subunits α and β of ATP synthase. Inhibition of GSK3 β by either phosphorylation by AKT or lithium chloride resulted in activation of pyruvate dehydrogenase, *i.e.*, decrease of its phosphorylated form. AKT-dependent phosphorylation of ATP synthase subunits α and β resulted in an increased complex activity. AKT translocation to mitochondria was associated with an increased expression and activity of complex I. These data suggest that the mitochondrial signaling pathway AKT-GSK3 β -PDH, AKT-dependent phosphorylation of ATP synthase, and upregulation of mitochondrial complex I expression and activity are involved in the control of mitochondrial bioenergetics by increasing substrate availability and regulating the mitochondrial catalytic/energy-transducing capacity.

Keywords

Mitochondrial respiration; PI3K; AKT; PTEN; GSK3 β ; pyruvate dehydrogenase; ATP synthase; hepatocytes; bioenergetics

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Introduction

Mitochondria are major cellular energy-transducing organelles that integrate metabolic processes and signaling pathways and are closely involved in the regulation of cell survival and apoptotic pathways. The phosphatidylinositol-3 kinase (PI3K) signaling pathway—activated by the binding of insulin, growth factors, and cytokines to the cell surface receptor [1]—regulates glucose and lipid metabolism [2], processes linked to transfer of reducing equivalents—generated by the tricarboxylic acid cycle—through the respiratory chain and coupled to oxidative phosphorylation. The enzymatic activity of PI3K leads to the conversion of phosphatidylinositol-4,5-phosphate (PIP₂) to phosphatidylinositol-3,4,5-phosphate (PIP₃) [3]. Binding of PIP₃ to pleckstrin homologue (PH) domains of downstream molecules serves as a major signaling node that transmits cell growth and hormone signals from cell surface to inside the cells [4, 5]. In muscle, insulin is a major regulator for mitochondrial ATP production partly by promoting the synthesis of resident mitochondrial genes and proteins [6, 7]. Consistent with a role of PI3K signaling in oxidative phosphorylation, deletion of forkhead transcriptional factor FoxO1 (downstream of PI3K) increased electron transport chain activity and normalized NAD⁺/NADH values in the IRS1/2 double knockout mouse liver [8]. In mouse models of resistance to insulin and activation of PI3K, such as the ob/ob mice [8, 9], mitochondrial electron-transfer chain activity and determinants of NAD⁺/NADH values are downregulated. Oxidative phosphorylation genes are also deregulated when liver cells fail to respond to insulin, such as in individuals with insulin resistance [8]. PI3K signaling also plays a key role in cell survival, partly due to its effect on regulating the release of mitochondrial cytochrome *c* [10]. Activation of PI3K [2] or inhibition of a negative regulator for the pathway, PTEN (Phosphatase and Tensin Homologue deleted on Chromosome 10) results in accelerated cell growth and better survival [11–14]. Insulin signals through PI3K to induce lipogenesis and glucose uptake/metabolism in both muscle and adipose tissues [15]. Deletion of PTEN results in improved insulin sensitivity, increased lipogenesis and glucose uptake/metabolism [16, 17].

A major target of PI3K activated by binding of PIP₃ is the serine/threonine kinase AKT [18, 19]. Activation of PI3K by insulin and other growth factors leads to phosphorylation and activation of AKT [18, 19]. PTEN removes the 3' phosphate moiety from PIP₃ and converts it to PIP₂ [20, 21], thereby inhibiting signaling transduction. In cells lacking PTEN, AKT is ubiquitously phosphorylated [12, 14, 16, 17, 22–25]. Activation of AKT and the consequential inhibition of FoxO lead to lipogenesis [17, 26, 27]. AKT was found to regulate the mitochondrial intrinsic cell death signaling through direct or indirect interactions with pro-apoptotic factors of the Bcl-2 family (*i.e.*, Bad and Bax) and glycogen synthase kinase 3 [10]. Bad and GSK3 are the two best-characterized substrates that are directly phosphorylated and inhibited by AKT [28]. Thus, glucose/lipid metabolism and cell survival/apoptosis—both regulated by PI3K/AKT signaling—converge at mitochondria.

How AKT may control mitochondrial respiratory function, however, is not clear. AKT may phosphorylate hexokinase II and strengthen its association with the outer mitochondrial membrane voltage-dependent anion channel (VDAC), thus activating the first step in glucose metabolism [28, 29]. AKT may have indirect effects on mitochondrial respiration in terms of increased substrate supply (pyruvate) to mitochondria as well as direct effects that entail the translocation of AKT to mitochondria [30, 31]. The latter is associated with an increased phosphorylation (inhibition) of a mitochondrial constitutive form of GSK3 β in neuroblastoma cells [31]. However, the biological function of AKT translocation and phosphorylation of mitochondrial GSK3 β is not clear. Association of AKT with other mitochondrial proteins, such as ATP synthase, was also reported [31].

This study examines the role of the PI3K/AKT pathway on mitochondrial bioenergetics by experimental approaches—performed on immortalized hepatocytes—entailing (a) genetic approaches (specific deletion of *Pten*), (b) stimulation of the PI3K/AKT route by IGF-1, and (c) specific inhibition of PI3K. The results indicate that AKT activation (phosphorylation) enhances cellular bioenergetics by mechanisms that increase both substrate supply to- and the catalytic efficiency of mitochondria.

Materials and Methods

Cell lines

Immortalized wild type control (Control) and *Pten* null (*Pten*^{−/−}) hepatocytes were established from 1-month old *Pten*^{loxP/loxP}; *Alb-Cre*[−] and *Pten*^{loxP/loxP}; *Alb-Cre*⁺ mouse liver, respectively [17, 25]. The cell lines were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 5 μg/ml insulin, 10 ng/ml EGF in a humidified 5% CO₂ atmosphere. Primary hepatocytes were cultured from 3-month old *Pten*/*Akt2* double mutant (Dm) (*Pten*^{loxP/loxP}; *Akt2*^{−/−}; *Alb-Cre*⁺), *Pten*^{−/−} (*Pten*^{loxP/loxP}; *Alb-Cre*⁺), and control (*Pten*^{loxP/loxP}; *Alb-Cre*[−]) mouse liver by collagenase perfusion.

Chemicals

Oligomycin, rotenone, and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were purchased from MP Medicals (Solon, OH). Lithium chloride, sodium pyruvate, D-(+)-Glucose, insulin-like growth factor 1 (IGF-1), LY294002, and protease/phosphatase inhibitor cocktail were from Sigma Chemical Co (St Louis, MO).

XF24 extracellular metabolic flux analysis

Control and *Pten*^{−/−} hepatocytes were seeded on XF24 plates at a density of 2×10⁴ cells/well. The plate was incubated in a humidified 5% CO₂ atmosphere for 24 h. For IGF-1 treatment, cells were further incubated in the serum free DMEM for 6 h followed by IGF-1 treatment for 1 h. For LY294002 treatment, cells were treated with 40 μM LY294002 dissolved in unbuffered DMEM for 1 h. In both IGF-1 and LY294002 treatments, unbuffered DMEM was used as control. Four basal measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were made followed by the addition of mitochondrial inhibitors. Four readings were made after the injection of each inhibitor. Three mitochondrial inhibitors were used: oligomycin (1 μM) was added to inhibit the ATP synthase followed by the measurement of ATP turn over, FCCP (1 μM) was added to uncouple the mitochondria followed by the measurement of the maximal mitochondrial respiratory capacity, and rotenone (1 μM) was added to inhibit the complex I followed by the measurement of non-mitochondrial respiration.

Mitochondria isolation

Mitochondria were isolated from control and *Pten*^{−/−} immortalized hepatocytes by using a Thermo Scientific Kit (Rockford, IL) by using dounce homogenization followed by centrifugation; the mitochondrial pellet was re-suspended in 15% Percoll and topped on to a pre-formed discontinuous Percoll gradient. The purified mitochondrial fraction was collected after centrifugation. The purity of mitochondrial fraction was assessed by measuring markers of microsomal (NADPH-cytochrome P450 reductase) and cytosolic (lactate dehydrogenase) contamination. In control hepatocytes, NADPH-cytochrome P450 reductase activity was 18.7 nmol/min/mg protein in the cell lysates and 1.19 nmol/min/mg protein in the mitochondrial fraction; lactate dehydrogenase activity was 16.2 μmol/min/mg protein in the cytosol and 2.17 μmol/min/mg protein in the mitochondrial fraction. The activity of lactate dehydrogenase in the cytosol of *Pten*^{−/−} cells was 25 μmol/min/mg protein

and that in the mitochondrial fraction, 3 $\mu\text{mol/min/mg}$ protein; NADPH-cytochrome P450 reductase activity in *Pten*^{-/-} cell lysates was 19 nmol/min/mg protein and in the mitochondrial fraction 2.5 nmol/min/mg protein.

Proteinase K treatment of mitochondria

Mitochondria from control and *Pten*^{-/-} hepatocytes were treated with proteinase K (50 $\mu\text{g/ml}$) at 4°C for 20 min. Phenylmethylsulfonyl fluoride (2 mM) was added to stop the reaction and mitochondria were garnered by centrifugation. Mitochondria were then lysed in 2% CHAPS containing protease and phosphatase inhibitors.

Western blot analysis

Control and *Pten*^{-/-} hepatocytes treated with various doses of IGF-1, LY294002, or LiCl were lysed in RIPA buffer (50 mM Tris/HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, phosphatase inhibitor and protease inhibitor cocktail). Mitochondrial proteins were extracted by incubating isolated mitochondria for 30 min at 4°C in 2% CHAPS containing protease and phosphatase inhibitors. Different amounts (10, 20, 30 or 60 μg) of cell lysates or mitochondrial proteins were loaded in each well of 10–12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel followed by electrophoresis and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The antibodies used for immunoblotting are anti-pAKT, anti-AKT, anti-pGSK3, and anti-GSK3 from Cell Signaling (Danvers, MA); anti-pPDH-E_{1 α} from EMD Millipore (Billerica, MA) and anti-PDH-E_{1 α} from MitoSciences (Eugene, OR). Band intensities were analyzed by the VersaDoc system (Bio-Rad, Hercules, CA).

Enzyme activity assay

Mitochondrial ATP synthase activity was measured by monitoring ATP hydrolysis (ATPase activity) in the reaction buffer containing 60 mM sucrose, 50 mM triethanolamine-HCl, 50 mM KCl, 4 mM MgCl₂, 2 mM ATP, 2 mM EGTA, 1 mM KCN (pH 8.0, adjusted by KOH) in the presence of 100 μM NADH, 5 units/ml pyruvate kinase and 5 units/ml lactate dehydrogenase at 37°C. The reaction was monitored for 5 min and the ATP synthase activity was determined by the changes of absorbance at 340 nm [32, 33]. Mitochondrial PDH activity was measured in PDH assay buffer (35 mM KH₂PO₄, 2 mM KCN, 0.5 mM EDTA, 5 mM MgCl₂, pH 7.25 with KOH) in the presence of 200 mM sodium pyruvate, 2.5 mM rotenone, 4 mM sodium CoA (co-enzyme A), 40 mM TPP (thiamine pyrophosphate) at 37°C. The conversion of NAD (nicotinamide adenine dinucleotide) to NADH (nicotinamide adenine dinucleotide reduced form) was monitored by the change of absorbance at 340 nm [34]. Activity for complexes I, II, and IV was determined using microplate assay kits (Abcam) in which the respective complexes were immunocaptured in the microplate wells, and the enzyme activity was determined by following the change in absorbance at 450, 600, and 550 nm, respectively.

Immunoprecipitation

Mitochondrial proteins (1 mg/ml) prepared from control and *Pten*^{-/-} immortalized hepatocytes were incubated with phospho-AKT-substrate (PAS) antibody conjugated beads (Cell Signaling) for 48 h. The beads with the immune complexes were washed 3 times with DPBS, mixed with reducing loading sample buffer (Thermo Scientific, Rockford, IL), and heated for 5 min at 100°C. Proteins were separated by a 12% SDS-PAGE gel. The gel was stained with Coomassie blue (Biorad, Hercules, CA). To detect the AKT contact and phosphorylation of ATP synthase, mitochondrial proteins (1 mg/ml) were immuno-precipitated with anti-phospho-AKT-substrate antibody conjugated beads and anti-AKT antibody plus protein A/G agarose beads respectively followed by SDS-PAGE

electrophoresis. The membrane is incubated with MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (MS604; Mitosciences, Eugene, OR) containing anti-Complex I subunit NDUFB8 (CI-20), anti-Complex II subunit 30kDa (CII-30), anti-Complex III subunit Core 2 (CIII-core2), anti-Complex IV subunit I (CIV-I), and anti-ATP synthase subunit alpha (CV-alpha).

LC/MS/MS analysis

Proteins were extracted from the Coomassie blue-stained gel followed by LC/MS/MS analysis at the USC Keck School of Medicine Proteomics Core Facility. In-gel trypsin digest: Protein bands from SDS-PAGE were excised from the gels using biopsy punches (Acuderm, Lauderdale, FL). In-gel tryptic digest was carried out using trypsin that was reductively methylated to reduce autolysis. The digestion reaction was carried out overnight at 37 °C. Digestion products were extracted from the gel with a 5% formic acid, 50% (v/v) acetonitrile solution (2×), and one acetonitrile extraction followed by evaporation using an APD SpeedVac (ThermoSavant, Milford, MA). Digested peptide mixture was analyzed by an online tandem LC/MS system. The LC/MS system consists of an Eksigent NanoLC-2D connected to an LTQ XL linear ion trap mass spectrometer equipped with a nanospray ion source (Thermo Fisher Scientific, Waltham, MA). Peptides were separated on a 10 cm reverses phase (RP) spray tip column connected to a 10 cm RP extended column through a MicroTee with in-union high voltage contact as described [35, 36]. Sample was loaded to the column using a 2 ml sample loop. Peptides were eluted at a flow rate of 500 nl/min into the mass spectrometer using the following gradients: 5–50% ACN + 0.1% formic acid over 75 minutes and 50–90% ACN + 0.1% formic acid over 40 min. The LTQ was operated at 2.6 kV spray voltage and 275°C capillary temperature. Full scan was generated and 10 most intensive ions above minimum signal threshold (500 counts) in each full scan were subjected for MS/MS fragmentation. MS/MS experiments were performed with normalized collision energy of 35% and isolation width of 2 m/z. The resulting spectra were searched against Uniprot protein database using SEQUEST in BioWorks Browser 3.2EF1. The results were filtered using Δcn and cross-correlation score (Xc) versus charge state. Δcn was always set at 0.1 and Xc was set at 1.50 for +1 charged peptides, 2.00 for +2 charged peptides, and 2.50 for +3 or higher charged peptides [35, 36].

Statistical analysis

Statistical analysis was performed using student's *t* test for unpaired data or ANOVA. $P < 0.05$ was considered significant.

Results

PI3K/AKT signaling activation induces mitochondrial respiration

Liver is a major organ where lipid and glucose metabolism converge with mitochondrial respiration. The role of PI3K/AKT signaling in mitochondrial respiration was assessed with a hepatocyte cell line established from mouse liver. Treatment of hepatocytes with insulin growth factor-1 (IGF-1)—that induces the activation of the PI3K/AKT pathway—at 50 and 100 ng/ml for 1 h induced a robust activation of AKT as indicated by the increase in its phosphorylation at Ser⁴⁷³ (Fig. 1A). A concomitant increase of the phosphorylated forms of GSK3 α and β at Ser²¹ and Ser⁹, respectively, indicated increased AKT activity in the IGF-1-treated samples (Fig. 1A).

IGF-1 treatment of hepatocytes resulted in an increase in oxygen consumption rates (OCR) and extracellular acidification rates (ECAR), the former reflecting mitochondrial respiration and the latter anaerobic glycolysis (*i.e.*, lactate formation). IGF-1 pre-treatment for 1 h did not affect the basal OCR, whereas it elicited a substantial increase of maximal respiratory

capacity (following the addition of the uncoupler FCCP) (Fig. 1B). At an IGF-1 concentration of 50 ng/ml, the maximal OCR increased by 90.61 ± 2.71 % (compared to the vehicle-treated cells) (Fig. 1B); a further increase was observed following treatment of hepatocytes with IGF-1 at a concentration of 100 ng/ml (128.18 ± 7.79 % higher than vehicle-treated cells) (Fig. 1B). IGF-1 treatment also increased ECAR (30.64 ± 8.64 % and 56.50 ± 5.78 % by 50 ng/ml and 100 ng/ml IGF-1, respectively) indicating an enhanced anaerobic glycolysis (Fig. 1D). These data suggest that IGF-1/PI3K/AKT signaling positively regulates anaerobic glycolysis and mitochondrial maximal respiratory capacity; up-regulation of glycolysis reflects increased substrate supply to mitochondria. Dranka *et al* [37] proposed an intermediate turnover state in cells (between state 3 and state 4 respiration), termed State_{apparent} that provides information on the mitochondrial workload and is calculated from the equation $\text{State}_{\text{apparent}} = 4 - [(\text{Basal} - \text{Oligo})/(\text{FCCP} - \text{Oligo})]$ [37, 38]; based on this equation, values of State_{apparent} for IGF-1-treated hepatocytes were 3.48 ± 0.03 and 3.62 ± 0.03 (for 50- and 100 ng IGF-1/ml, respectively). Likewise, the mitochondrial reserve capacity [37, 38] was substantially increased by IGF-1 treatment (50 ng/ml, 134.30 ± 11.02 pmoles/min; 100 ng/ml, 206.75 ± 19.21 pmoles/min). The low maximal respiratory capacity value (lower than basal OCR) in vehicle-treated hepatocytes was due to low level of active AKT after the 6 h incubation in serum-free medium (a requirement when treating cells with IGF-1) (Fig. 1 A). The data indicate that AKT activation is critical to stimulate and maintain the mitochondrial reserve capacity (measured by the addition of mitochondrial uncoupler, FCCP). When immortalized control hepatocytes are starved with serum free medium for 6 h, AKT phosphorylation level is low and there is almost no reserve capacity. The cell density in the IGF-1 treatment groups was the same as that in the vehicle group. The reserve capacity was restored in the presence of IGF-1.

Inhibition of PI3K/AKT signaling attenuates mitochondrial respiration

Inhibition of PI3K by LY294002 resulted in a decreased AKT and GSK3 phosphorylation (Fig. 2A), the former indicating a lack of activation and the latter release of the inhibitory effect exerted upon phosphorylation at Ser⁹ of GSK3 β . Accordingly, basal and maximal OCRs were reduced (38.8 ± 4.6 and 50.6 ± 2.9 , respectively) by treatment with LY294002 as compared with vehicle-treated cells (Fig. 2B,C). Decreased basal and maximal OCR indicates that the inhibition of the PI3K/AKT signaling by LY294002 treatment leads to the inhibition of coupled and maximal mitochondrial respiration. The inhibition of ECAR by LY294002 treatment implied that anaerobic glycolysis was not compensating for the decrease in mitochondrion-driven O₂ consumption (Fig. 2D). LY294002 treatment decreased the mitochondrial reserve capacity [37, 38] from 161.53 ± 27.51 pmoles/min to 59.87 ± 11.71 pmoles/min and S_{apparent} from 3.64 ± 0.05 to 3.51 ± 0.08). These data, along with those following IGF-1 treatment, strengthen the importance of PI3K/AKT signaling on mitochondrial bioenergetics.

Mitochondrial bioenergetics in *Pten*^{-/-} immortalized hepatocytes

An isogenic murine hepatocyte cell line with constitutive activation of PI3K/AKT signaling was used in order to confirm the role of PI3K/AKT in mitochondrial respiration. The *Pten* null (*Pten*^{-/-}) hepatocyte cell line was established from a 1 month-old mouse carrying a liver-specific deletion of *Pten* (*Pten*^{loxP/loxP}; *Alb-Cre*⁺) [25] (Fig. 3A). Immunoblotting analysis revealed that AKT activity—indicated by its phosphorylation status—was substantially increased in the *Pten*^{-/-} hepatocytes as compared with the control cell lines with intact *Pten* (Fig. 3B). Accordingly, GSK3 β phosphorylation at Ser⁹ was increased (Fig. 3B). Fig. 3C shows a time course of AKT and GSK3 α and GSK3 β phosphorylation in control- and *Pten*^{-/-} hepatocytes following PI3K inhibition by LY294002.

Basal OCR was significantly higher in the *Pten*^{-/-} than in the control hepatocytes (289.76 ± 3.74 and 154.88 ± 2.88 pmoles/min, respectively). Following the addition of FCCP, maximal hepatocytes (393.32 ± 56.64 pmoles/min) remained significantly higher than OCR in the *Pten*^{-/-} than in the controls (213.50 ± 8.14 pmoles/min) (Fig. 4 A,B). The number of control cells and *Pten*^{-/-} cells in Fig. 4A was the same, as indicated by similar values of non-mitochondrial respiration (following the addition of rotenone) and measurements of protein concentration in both cell types at the end of the experiment. Basal ECAR was higher in the *Pten*^{-/-} hepatocytes (40.48 ± 1.43 mpH/min) than in the controls (26.99 ± 1.99 mpH/min). The higher anaerobic glycolysis level in *Pten*^{-/-} hepatocytes indicates a higher substrate supply from glycolysis to mitochondria. Compared with control hepatocytes, *Pten*^{-/-} hepatocytes showed an enhanced mitochondrial reserve capacity (108.8 ± 11.02 pmoles/min) than control hepatocytes (38.35 ± 22.16 pmoles/min; *Pten*^{-/-}). Taken together, these data confirmed that the PTEN-regulated PI3K/AKT pathway controls bioenergetics by modulating both substrate supply (ECAR) and mitochondrial catalytic/energy transducing capacity (OCR).

The involvement of AKT in the regulation of cellular bioenergetics was further confirmed genetically in primary hepatocytes from a double knockout (*Pten*^{-/-}, *Akt2*^{-/-}) model, which show a reduced level of basal OCR (312.18 ± 18.64 pmoles/min) as compared with *Pten*^{-/-} hepatocytes (424.71 ± 16.34 pmoles/min) (not shown). The slight decrease of maximal OCR in *Pten*^{-/-}, *Akt2*^{-/-} hepatocytes (480.82 ± 29.55 pmoles/min) compared with *Pten*^{-/-} hepatocytes (507.19 ± 50.95 pmoles/min) was not statistically significant. An explanation for this observation is that AKT2 mainly regulates the ATP synthase activity and no difference can be observed in the presence of FCCP (not shown).

Activated AKT translocates to mitochondria

Consistent with previous reports on the translocation of AKT to mitochondria in NIH/3T3 [30] and neuroblastoma [31] cells, a small amount of AKT translocated to mitochondria in both control- and *Pten*^{-/-} hepatocytes. *Pten*^{-/-} hepatocytes—with a more robust AKT phosphorylation—showed significantly more AKT in mitochondria (Fig. 5A). Expectedly, IGF-1 treatment also induced a higher translocation of AKT to mitochondria. This is more evident in the *Pten*^{-/-} cell lines, in which the pAKT/AKT ratio is already high without IGF-1 treatment. When treated with IGF-1, even more AKT translocates to the mitochondria in the *Pten*^{-/-} hepatocytes (Fig. 5B). Fig. 5C shows a time-dependent increase in pAKT (Ser⁴⁷³) levels in both control and *Pten*^{-/-} cell lines in response to IGF-1 treatment; compared with control, *Pten*^{-/-} cell lines showed significantly higher increase in pAKT (Ser⁴⁷³) levels in response to IGF-1.

Treatment of isolated mitochondria with proteinase K (that degrades proteins on the outside of the outer mitochondrial membrane) revealed that AKT translocates to mitochondria and localizes in mitochondrial inter-membrane space and mitochondrial matrix (Fig. 5D,E). The sensitivity of TOM20 (a mitochondrial outer-membrane protein) to proteinase K indicates that the experimental condition (incubation with 50 µg/ml of proteinase K for 20 min at 4°C) is sufficient to degrade mitochondrial outer-membrane proteins.

AKT may regulate mitochondrial respiration through GSK3β and ATP synthase

GSK3β which was proposed as a constitutive mitochondrial protein, is a well-characterized AKT phosphorylation target in these organelles [31]. GSK3β was shown to catalyze the phosphorylation (and inactivation) of the pyruvate dehydrogenase complex (PDH) [39], a critical mitochondrial enzyme complex that couples anaerobic glycolysis (in cytosol) to the oxidative decarboxylation of pyruvate to acetyl-CoA and its entry in the tricarboxylic acid cycle. Thus, GSK3β is a potential target of mitochondrial AKT that may be partly

responsible for PI3K/AKT induced mitochondrial respiration. Treatment of hepatocytes were treated with LiCl, a reagent commonly used to inhibit GSK3 β activity [40], led to increased phosphorylation (inactivation) of GSK3 β (Fig. 6A) and a dose-dependent reduction in phosphorylation (inactivation) of PDH-E₁ α (Fig. 6B), indicating activation of the enzyme complex. Accordingly, LiCl treatment increased mitochondrial PDH activity in both control (Fig. 7A) and *Pten*^{-/-} hepatocytes (Fig. 7B). Consistent with the role of PDH as a crucial mitochondrial enzyme, LiCl treatment increased basal and maximal respiration in control (Fig. 8A) and *Pten*^{-/-} hepatocytes (Fig. 8B). This observation supported a potential role for a mitochondrial AKT-GSK3 β -PDH signaling circuitry in regulating mitochondrial basal and maximal respiration. Consistent with the role of GSK3 β in PI3K/AKT signaling, the mitochondrial reserve capacity in *Pten*^{-/-} hepatocytes increased from 45.25 ± 34.65 pmoles/min to 330.33 ± 96.03 pmoles/min with LiCl treatment; no significant differences were observed in the mitochondrial reserve capacity in control hepatocytes in response to LiCl treatment. Thus, mitochondrial respiration may be regulated by the coordinated balance between GSK3 β (inhibitory effect) and AKT (stimulatory effect). In accordance with the increased phosphorylation level of GSK3 β at Ser⁹ (inactive), phosphorylation level of mitochondrial PDH-E₁ α at Ser²⁹³ (inactive) decreased in *Pten*^{-/-} hepatocytes (Fig. 9A), correlates with the enhanced PDH activity (Fig. 9B). Therefore, these data confirm that the AKT signaling affects mitochondrial bioenergetics in part through GSK3 β regulation of mitochondrial PDH.

Other potential mitochondrial AKT phosphorylation targets were examined by pull-down experiments from mitochondrial lysates prepared from control- and *Pten*^{-/-} hepatocytes. Immuno-precipitation with pAKT substrate antibody identified 4 bands at ~100, 90, 80, 50 kDa that were significantly stronger in *Pten*^{-/-} mitochondrial lysates than in the controls (Fig. 10A). LC/MS/MS analyses revealed several potential targets for phosphorylation by AKT (Table 1): the 100 kDa band was identified as leucine-rich PPR motif-containing protein; the 90 kDa band was identified as monofunctional C1-tetrahydrofolate synthase, lon protease, and NAD(P) transhydrogenase; the 80 kDa band as aconitase, and the 50 kDa band as glutamate dehydrogenase 1, aldehyde dehydrogenase, and ATP synthase subunit α and subunit β . Consistent with these observations, ATP synthase activity was higher in the *Pten*^{-/-} hepatocytes than in control cells (Fig. 10B).

ATP synthase subunit α contains the conserved sequence to the consensus RxRxxS/T sequence for a potential AKT substrate (Table 2). This sequence is located at a structure characteristic where the subunit α of ATP synthase may interact with the β subunit (Fig. 10C). Sequence analysis for ATP synthase α revealed that the consensus sequence segment is conserved in several species analyzed including human, mouse, rat, and *E. coli*. ATP synthase β has similar sequence features as a substrate for AKT (Table 2) and had been previously shown to be a potential substrate for AKT [31]. These analyses suggest that the ATP synthase enzymes are likely substrates for AKT and that their phosphorylation might be partly responsible for the higher OCR measurements after FCCP treatment in the *Pten*^{-/-} and IGF-1 treated cultured cells.

The assumption that AKT contacts and phosphorylates ATP synthase α was confirmed by immuno-precipitation and western-blotting analysis (Fig. 11A). Using a phospho-AKT substrate antibody (Fig. 11A, lanes 1 and 2) and total AKT antibody (Fig. 11A, lanes 3 and 4) to immune-precipitate substrates for AKT from the mitochondrial lysate followed by immunoblotting, a clear association of complex V ATP synthase 5 α with AKT was observed. The levels of ATP synthase 5 α associated with AKT were higher in mitochondria from *Pten*^{-/-} (Fig. 11A, lane 4) than those from control hepatocytes (Fig. 11A, lane 3).

In addition, complex I expression was higher in mitochondrial lysate from *Pten*^{-/-} cells (Fig. 11A, lane 6) than that from control hepatocytes (Fig. 11A, lane 5). This was further confirmed with immunoblotting of the respiratory chain complexes (Fig. 11B) showing a higher expression of complex I in the mitochondrial fraction from *Pten*^{-/-} cells than that from control hepatocytes; furthermore, complex I activity was significantly higher in *Pten*^{-/-} hepatocytes (Fig. 11C); no statistically significant changes were observed in complex II and complex IV activities. Thus, AKT activation is associated an increased mitochondrial electron transfer capacity independent of its direct kinase activity in mitochondria. A transcriptional regulation may be involved in this mechanism.

Discussion

The PI3K/AKT signaling pathway regulates both growth/survival and metabolism; however, it is not clear how these two functions are integrated. One potential link may be surmised to be integrated in mitochondria, organelles involved energy-transducing process and the regulation of apoptotic and survival pathways. These functions render mitochondria viable candidates for integrating the PI3K/AKT signaling pathway as well as other signaling pathways. This study showed that PI3K/AKT signaling positively regulates mitochondrial bioenergetics in experimental models entailing IGF-1-driven stimulation of the PI3K/AKT route (Fig. 1), inhibition of PI3K (Fig. 2), and a *Pten*^{-/-} cell model with a robust insulin signaling (Figs. 3,4).

The increased mitochondrial respiratory function correlated with the inhibition of GSK3 β by AKT. Inhibition of GSK3 β by LiCl exerted a decrease in phosphorylation (inactivation) of PDH-E₁ α at Ser²⁹³ and an increased mitochondrial basal and maximal respiration, indicating that GSK3 β negatively regulates mitochondrial bioenergetics through modulating PDH activity. AKT, therefore, may partly regulate mitochondrial bioenergetics through inhibiting GSK3 β . In rat hippocampal neurons, GSK3 β was found to be a mitochondrial resident protein that phosphorylated and inhibited PDH [39]. It may be surmised that mitochondrial pAKT phosphorylates and inhibits GSK3 β , thus changing the pPDH_{inactive}/PDH_{active} ratio. This function of AKT may represent a feed forward reaction that prevents the product of cytosolic glycolysis and mitochondrial PDH substrate–pyruvate–from buildup while simultaneously AKT activation induces glucose uptake, hexokinase-2/VDAC interaction, and glucose metabolism to pyruvate. PDK1, the enzyme that phosphorylates AKT on the second site Thr³⁰⁸ to achieve full activation, has been shown previously to be constitutively active in the mitochondrial inter-membrane space [30]. This observation further confirmed a role of PDK1-AKT-GSK3 β signaling axis in regulating mitochondrial function. However, stress conditions and aging are associated with translocation of JNK to mitochondria in primary cortical neurons [41, 42]; at variance with AKT (this study and [30, 31]), JNK associated with the outer mitochondrial membrane (proteinase K-sensitive) and triggered a phosphorylation cascade that involved PDK1 and resulted in phosphorylation and inhibition of PDH [41, 42].

The increased mitochondrial bioenergetics may also be attributed to the translocation of pAKT to mitochondria where it phosphorylates a number of other mitochondrial proteins including the two isoforms of ATP synthase, α and β . ATP synthase is a multi-subunit complex composed by an ATPase and a proton channel and it converts the membrane potential generated by the proton gradient to ATP. The α and β subunits are part of the ATPase complex and function as dimers; the sequence of ATP synthase, subunit α phosphorylated by AKT is part of the α - β interacting interfaces and its phosphorylation correlates with an increased ATPase activity. These observations imply that AKT-mediated phosphorylation of ATP synthase α affects its ability to interact with ATP synthase β and thus may affect directly its function. Though not an AKT phosphorylation substrate,

complex I expression and activity were increased in mitochondrial lysates from *Pten*^{-/-} cells (as compared to control hepatocytes). How a robust PI3K/AKT signaling and the effects of AKT translocation to mitochondria affect complex I expression and activity remains to be elucidated.

Recent evidence suggests that the ‘Warburg effect’ (enhanced glycolytic rates in aerobic conditions) is a hallmark of tumor cells and may contribute to the cause of tumorigenesis [43]. Upregulation of PI3K/AKT signaling—particularly because loss of PTEN function—is a common occurrence in human tumor specimens. However, how metabolic changes observed as Warburg effects contribute to this process are still unclear. The PI3K/AKT signaling pathway may be viewed as a major candidate accounting for some of the Warburg effects observed in cancer cells due to its dual role in growth and metabolism [2]. Transfection of Rat1a cells with a constitutively active form of AKT robustly increased the association of hexokinase with outer mitochondrial membrane VDAC [44], an effect that was critical for the anti-apoptotic function of AKT [45]. The ability of AKT to inhibit apoptosis is dependent on this association of hexokinase with the mitochondrial membrane [44, 45]. These studies, thus, suggest that mitochondria are critical for linking the metabolic and cell survival effects of PI3K/AKT signaling.

Taken together, this study demonstrated that PI3K/AKT activation leads to increased mitochondrial respiration through: 1) increasing substrate supply to mitochondria (regulation of cytosolic processes by AKT); 2) enhancing the mitochondrial catalytic machinery (changes in pPDH/PDH ratios and a stronger metabolism of pyruvate (PDH activity)) upon inhibition of GSK3 β and 3) up-regulation of mitochondrial electron transfer and energy-transduction capacity, the former may entail the increased expression level and activity of complex I whereas the latter may involve a direct phosphorylation of ATP synthase by AKT.

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Abbreviations

AKT	protein kinase B
COX IV	cytochrome <i>c</i> oxidase subunit IV
ECAR	extracellular acidification rate
EGF	epidermal growth factor
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
GSK3	glycogen synthase kinase 3
IGF-1	insulin-like growth factor-1
IRS	insulin receptor substrate
OCR	oxygen consumption rate
PDH	pyruvate dehydrogenase
PI3K	phosphatidylinositol-3-kinase
PTEN	phosphatase and tensin homolog deleted on Chromosome 10
VDAC	voltage-dependent anion channel

References

1. Wymann MP, Marone R. Phosphoinositide 3-kinase in disease: timing, location, and scaffolding. *Curr Opin Cell Biol.* 2005; 17:141–149. [PubMed: 15780590]
2. Cantley LC. The phosphoinositide 3-kinase pathway. *Science.* 2002; 296:1655–1657. [PubMed: 12040186]
3. Whitman M, Downes CP, Keeler M, Keller T, Cantley L. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature.* 1988; 332:644–646. [PubMed: 2833705]
4. Harlan JE, Hajduk PJ, Yoon HS, Fesik SW. Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. *Nature.* 1994; 371:168–170. [PubMed: 8072546]
5. Klarlund JK, Guilherme A, Holik JJ, Virbasius JV, Chawla A, Czech MP. Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. *Science.* 1997; 275:1927–1930. [PubMed: 9072969]
6. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman GI. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science.* 2003; 300:1140–1142. [PubMed: 12750520]
7. Shelley P, Martin-Gronert MS, Rowleson A, Poston L, Heales SJ, Hargreaves IP, McConnell JM, Ozanne SE, Fernandez-Twinn DS. Altered skeletal muscle insulin signaling and mitochondrial complex II–III linked activity in adult offspring of obese mice. *Am J Physiol Regul Integr Comp Physiol.* 2009; 297:R675–R681. [PubMed: 19535678]
8. Cheng Z, Guo S, Copps K, Dong X, Kollipara R, Rodgers JT, Depinho RA, Puigserver P, White MF. Foxo1 integrates insulin signaling with mitochondrial function in the liver. *Nature Med.* 2009; 15:1307–1311. [PubMed: 19838201]
9. Vianna CR, Huntgeburth M, Coppari R, Choi CS, Lin J, Krauss S, Barbatelli G, Tzameli I, Kim YB, Cinti S, Shulman GI, Spiegelman BM, Lowell BB. Hypomorphic mutation of PGC-1 β causes mitochondrial dysfunction and liver insulin resistance. *Cell Metab.* 2006; 4:453–464. [PubMed: 17141629]
10. Stiles BL. PI-3-K and AKT: Onto the mitochondria. *Adv Drug Deliv Rev.* 2009; 61:1276–1282. [PubMed: 19720099]
11. Galicia VA, He L, Dang H, Kanel G, Vendryes C, French BA, Zeng N, Bayan JA, Ding W, Wang KS, French S, Birnbaum MJ, Rountree CB, Stiles B. Expansion of hepatic tumor progenitor cells in Pten-null mice requires liver injury and is reversed by loss of AKT2. *Gastroenterology.* 2010; 139:2170–2182. [PubMed: 20837017]
12. Stiles B. Essential role of AKT-1/protein kinase B α in PTEN-controlled tumorigenesis. *Mol Cell Biol.* 2002; 22:3842–3851. [PubMed: 11997518]
13. Stiles B, Groszer M, Wang S, Jiao J, Wu H. PTENless means more. *Dev Biol.* 2004; 273:175–184. [PubMed: 15328005]
14. Stiles BL, Kuralwalla-Martinez C, Guo W, Gregorian C, Wang Y, Tian J, Magnuson MA, Wu H. Selective deletion of Pten in pancreatic beta cells leads to increased islet mass and resistance to STZ-induced diabetes. *Mol Cell Biol.* 2006; 26:2772–2781. [PubMed: 16537919]
15. Khan CR. The molecular mechanism of insulin action. *Annu Rev Med.* 1985; 36:429–451. [PubMed: 2986528]
16. Kurlawalla-Martinez C, Stiles B, Wang Y, Devaskar SU, Kahn BB, Wu H. Insulin hypersensitivity and resistance to streptozotocin-induced diabetes in mice lacking PTEN in adipose tissue. *Mol Cell Biol.* 2005; 25:2498–2510. [PubMed: 15743841]
17. Stiles B, Wang Y, Stahl A, Bassilian S, Lee WP, Kim YJ, Sherwin R, Devaskar S, Lesche R, Magnuson MA, Wu H. Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity. *Proc Natl Acad Sci USA.* 2004; 101:2082–2087. [PubMed: 14769918]
18. Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell.* 1995; 81:727–736. [PubMed: 7774014]

19. Kohn AD, Takeuchi F, Roth RA. Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J Biol Chem.* 1996; 271:21920–21926. [PubMed: 8702995]
20. Hopkin K. A surprising function for the PTEN tumor suppressor. *Science.* 1998; 282:1027–1230. [PubMed: 9841444]
21. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem.* 1998; 273:13375–13378. [PubMed: 9593664]
22. Rountree CB, Ding W, He L, Stiles B. Expansion of CD133-expressing liver cancer stem cells in liver-specific phosphatase and tensin homolog deleted on chromosome 10-deleted mice. *Stem Cells.* 2009; 27:290–299. [PubMed: 19008348]
23. Stanger BZ, Stiles B, Lauwers GY, Bardeesy N, Mendoza M, Wang Y, Greenwood A, Cheng KH, McLaughlin M, Brown D, Depinho RA, Wu H, Melton DA, Dor Y. Pten constrains centroacinar cell expansion and malignant transformation in the pancreas. *Cancer Cell.* 2005; 8:185–195. [PubMed: 16169464]
24. Stiles BL. Phosphatase and tensin homologue deleted on chromosome 10: extending its PTENacles. *Int J Biochem Cell Biol.* 2009; 41:757–761. [PubMed: 18950730]
25. Xu X, Kobayashi S, Qiao W, Li C, Xiao C, Radaeva S, Stiles B, Wang RH, Ohara N, Yoshino T, LeRoith D, Torbenson MS, Gores GJ, Wu H, Gao B, Deng CX. Induction of intrahepatic cholangiocellular carcinoma by liver-specific disruption of Smad4 and Pten in mice. *J Clin Invest.* 2006; 116:1843–1852. [PubMed: 16767220]
26. He L, Hou X, Kanel G, Zeng N, Galicia V, Wang Y, Yang J, Wu H, Birnbaum MJ, Stiles BL. The critical role of AKT2 in hepatic steatosis induced by PTEN loss. *Am J Pathol.* 2010; 176:2302–2308. [PubMed: 20348245]
27. Leavens KF, Easton RM, Shulman GI, Previs SF, Birnbaum MJ. Akt2 is required for hepatic lipid accumulation in models of insulin resistance. *Cell Metab.* 2009; 10:405–418. [PubMed: 19883618]
28. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell.* 1997; 91:231–241. [PubMed: 9346240]
29. Pastorino JG, Hoek JB. Ethanol potentiates tumor necrosis factor-alpha cytotoxicity in hepatoma cells and primary rat hepatocytes by promoting induction of the mitochondrial permeability transition. *Hepatology.* 2000; 31:1141–1152. [PubMed: 10796891]
30. Antico Arciuch VG, Galli S, Franco MC, Lam PY, Cadenas E, Carreras MC, Poderoso JJ. Akt1 intramitochondrial cycling is a crucial step in the redox modulation of cell cycle progression. *PLoS One.* 2009; 4:e7523. [PubMed: 19844585]
31. Bijur GN, Jope RS. Rapid accumulation of Akt in mitochondria following phosphatidylinositol 3-kinase activation. *J Neurochem.* 2003; 87:1427–1435. [PubMed: 14713298]
32. Das AM. Regulation of mitochondrial ATP synthase activity in human myocardium. *Clin Sci (Lond).* 1998; 94:499–504. [PubMed: 9682672]
33. Rosing J, Harris DA, Kemp AJ, Slater EC. Nucleotide-binding properties of native and cold-treated mitochondrial ATPase. *Biochim Biophys Acta.* 1975; 376:13–26. [PubMed: 123764]
34. Gohil K, Jones DA. A sensitive spectrophotometric assay for pyruvate dehydrogenase and oxoglutarate dehydrogenase complexes. *Biosci Rep.* 1983; 3:1–9. [PubMed: 6839008]
35. Wang LC, Okitsu CY, Kochounian CH, Rodriguez A, Hsieh CL, Zandi E. A simple and inexpensive on-column frit fabrication method for fused-silica capillaries for increased capacity and versatility in LC-MS/MS applications. *Proteomics.* 2008; 8:1758–1761. [PubMed: 18384106]
36. Garcia J, Han D, Sancheti H, Yap LP, Kaplowitz N, Cadenas E. Regulation of mitochondrial glutathione redox status and protein glutathionylation by respiratory substrates. *J Biol Chem.* 2010; 285:39646–39654. [PubMed: 20937819]
37. Dranka BP, Benavides GA, Diers AR, Giordano S, Zelikson BR, Reily C, Zou L, Chatham JC, Hill BG, Zhang J, Landar A, Darley-Usmar VM. Assessing bioenergetic function in response to oxidative stress by metabolic profiling. *Free Radic Biol Med.* 2011; 51:1621–1635. [PubMed: 21872656]

38. Sansbury BE, Jones SP, Riggs DW, Darley-Usmar VM, Hill BG. Bioenergetic function in cardiovascular cells: the importance of the reserve capacity and its biological regulation. *Chem Biol Interact.* 2011; 191:288–295. [PubMed: 21147079]
39. Hoshi M, Takashima A, Noguchi K, Murayama M, Sato M, Kondo S, Saitoh Y, Ishiguro K, Hoshino T, Imahori K. Regulation of mitochondrial pyruvate dehydrogenase activity by tau protein kinase I/glycogen synthase. kinase 3beta in brain. *Proc Natl Acad Sci USA.* 1996; 93:2719–2723. [PubMed: 8610107]
40. Jope RS. Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes. *Trends Pharmacol Sci.* 2003; 24:441–443. [PubMed: 12967765]
41. Zhou Q, Lam PY, Han D, Cadenas E. c-Jun N-terminal kinase regulates mitochondrial bioenergetics by modulating pyruvate dehydrogenase activity in primary cortical neurons. *J Neurochem.* 2008; 104:325–335. [PubMed: 17949412]
42. Zhou Q, Lam PY, Han D, Cadenas E. Activation of c-Jun-N-terminal kinase and decline of mitochondrial pyruvate dehydrogenase activity during brain aging. *FEBS Lett.* 2009; 583:1132–1140. [PubMed: 19272379]
43. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science.* 2009; 324:1029–1033. [PubMed: 19460998]
44. Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev.* 2001; 15:1406–1418. [PubMed: 11390360]
45. Majewski N, Nogueira V, Bhaskar P, Coy PE, Skeen JE, Gottlob K, Chandel NS, Thompson CB, Robey RB, Hay N. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Mol Cell.* 2004; 16:819–830. [PubMed: 15574336]

Highlights

- PI3K/AKT signaling modulates both substrate availability and mitochondrial energy-transducing capacity.
- Translocation of AKT to mitochondria and identification of several mitochondrial targets of AKT phosphorylation, including ATP synthase subunits α and β .
- AKT translocation to mitochondria was associated with increased activities of pyruvate dehydrogenase, mitochondrial respiratory complex I, and ATP synthase activities.

Fig. 1. Effect of IGF-1 on AKT phosphorylation status and cellular bioenergetics

(A) Western blots of AKT and GSK3 and their phosphorylated forms. Hepatocytes were incubated in serum-free medium for 6 h and treated with IGF-1 for 1 h. β -actin was used as protein loading controls. (B) Effect of IGF-1 on oxygen consumption rate (OCR). Immortalized hepatocytes (2×10^4 cells/well) were pre-incubated with IGF-1 for 1 h before starting the measurement of basal respiration. Subsequent additions: 1 μ M oligomycin; 1 μ M FCCP; 1 μ M rotenone. Four readings were made after the injection of each inhibitor. (●) vehicle; (●) plus 50 ng IGF-1/ml; (●) plus 100 ng IGF-1/ml. (C) Quantitation of basal and maximum OCR in vehicle- and IGF-1-treated samples (data from Fig. 1C). Data are the average of 4 time-points each from 4 independent samples. (D) Effect of IGF-1 on extracellular acidification rate (ECAR): assay conditions as in Fig. 1B. All experiments were performed in quadruplicate. Other assay conditions as described in the Materials and Methods section. ** $P < 0.01$.

Fig. 2. Effect of PI3K inhibition on AKT and GSK phosphorylation status and cellular bioenergetics

(A) Western blots of AKT and GSK3α/β and their phosphorylated forms. Cells were incubated with 40 μM LY294002 for 1 h. β-actin is detected as protein loading controls. (B) Effect of PI3K inhibitor on oxygen consumption rate (OCR) (assay performed on 2×10^4 cells/well). Basal OCR rate was measured 30 min after the addition of LY294002. (●) vehicle; (●) plus 40 μM LY294002. (C) Quantitation of basal and maximal OCR in vehicle- and LY294002-treated samples. (D) Effect of LY294002 on extracellular acidification rate (ECAR); assay conditions as in Fig. 2B. All experiments were performed in quadruplicates. Quantitative data in (C) are average of 4 time-points each from 4 independent samples. ** $P < 0.01$.

Fig. 3. AKT and GSK3 phosphorylation status in immortalized hepatocytes from wild- and *Pten*^{-/-} knockout mice

(A) PTEN protein expression in control- and *Pten*^{-/-} hepatocytes. (B) Western blots of AKT and GSK3 and their phosphorylated forms in cell lysates from control- and *Pten*^{-/-} hepatocytes. Different amounts of proteins (10-, 30-, and 60 μg) were loaded in three sets of samples. (C) Effect of LY294002 on the time course of p-AKT, p-GSK3α, and p-GSK3β in control- and *Pten*^{-/-} hepatocytes. β-actin as protein loading controls. Densitometry reading of p-AKT to β-actin ratio (pAKT/β-actin) for each lane is shown at the bottom of the blots.

Fig. 4. Bioenergetics of control- and *Pten*^{-/-} hepatocytes

(A) Oxygen consumption rates (OCR) of control- and *Pten*^{-/-} hepatocytes (2×10^4 cells/well). Additions (oligomycin, FCCP, and rotenone) as in Fig. 1B. (●) control hepatocytes; (●) *Pten*^{-/-} hepatocytes. (B) Quantitative data of basal and maximal OCR of control (●)- and *Pten*^{-/-} (●) hepatocytes (data from Fig. 4A). (C) Extracellular acidification rate (ECAR) of control- and *Pten*^{-/-} hepatocytes (2×10^4 cells/well) (assay conditions as in Fig. 4A). (D) Quantitative data of basal ECAR of control- and *Pten*^{-/-} hepatocytes. All experiments were performed in quadruplicate. Data in (B) and (D) are the average of 4 time-points from 4 different samples. ** $P < 0.01$.

Fig. 5. Mitochondrial localization of AKT

(A) AKT expression in the mitochondrial fraction isolated from control and *Pten*^{-/-} hepatocytes. (B) Time course of AKT expression in the mitochondrial fraction following incubation of hepatocytes with 50 ng IGF-1/ml. Cells (control and *Pten*^{-/-} hepatocytes) were incubated for 0, 5, 15, and 30 min previous to the isolation of the mitochondrial fraction. (C) Time course p-AKT expression in the cell lysate following incubation for different times with 50 ng IGF-1/ml. L, light exposure; D, dark exposure. (D) Effect of proteinase K on AKT and p-AKT expression in the mitochondrial fraction isolated from control and *Pten*^{-/-} hepatocytes. TOM20, a mitochondrial outer membrane protein was used as control for proteinase K activity. (E) Relative levels of mitochondrial AKT in control and *Pten*^{-/-} hepatocytes (data from (D)). COX IV, an inner membrane protein is used for control of mitochondrial protein loading and β-actin used for total cell lysate loading.

Fig. 6. Effect of LiCl on p-GSK3 β and p-PDH in the cell lysate and mitochondrial fraction, respectively

(A) Effect of different concentrations of LiCl on p-GSK3 β (Ser⁹) and GSK3 β expression. Hepatocytes were treated with the different concentrations of LiCl for 4 h. β -actin as protein loading controls. (B) Effect of different concentrations of LiCl on p-PDH-E₁ α (Ser²⁹³) and PDH-E₁ α expression. COX IV, an inner membrane protein is used for control of mitochondrial protein loading.

Fig. 7. Effects of LiCl treatment on mitochondrial PDH activity in control and *Pten*^{-/-} immortalized hepatocytes

The effects of increasing concentration of LiCl on mitochondrial PDH activity in control (A) hepatocytes and *Pten*^{-/-} (B) hepatocytes. Hepatocytes were incubated with difference concentrations (10 and 20 mM) of LiCl for 4h. Mitochondria were isolated after LiCl treatment and PDH activity was measured as described. **P < 0.01.

Fig. 8. Effect of LiCl on the bioenergetics of control- and *Pten*^{-/-} hepatocytes

(A) Effect of LiCl on the oxygen consumption rate (OCR) of control hepatocytes. Cells (2×10^4 /well) were incubated for 4 h with 20 mM LiCl before starting the measurement of basal respiration. Additions (oligomycin, FCCP, and rotenone) as in Fig. 1B. Graph on the right: quantitative data indicating the effect of LiCl on basal- and maximal respiration. (B) Effect of LiCl on the oxygen consumption rate (OCR) of *Pten*^{-/-} hepatocytes. Assay conditions as in (A). Graph on the right: quantitative data indicating the effect of LiCl on basal- and maximal respiration of *Pten*^{-/-} hepatocytes. ** $P < 0.01$.

Fig. 9. Pyruvate dehydrogenase phosphorylation status and activity in mitochondria from control and *Pten*^{-/-} immortalized hepatocytes

(A) Western blots of mitochondrial pyruvate dehydrogenase and its phosphorylated form (Ser²⁹³) in control- and *Pten*^{-/-} immortalized hepatocytes. Mitochondria were isolated from control- and *Pten*^{-/-} immortalized hepatocytes. 20 μ g mitochondrial proteins were loaded in each well; (B) Measurement of PDH activity in mitochondria from control- and *Pten*^{-/-} immortalized hepatocytes. (●)control hepatocytes; (●)*Pten*^{-/-} hepatocytes. **P < 0.01.

Fig. 10. AKT phosphorylation targets in mitochondrial fraction isolated from control- and *Pten*^{-/-} hepatocytes

(A) Coomassie blue staining of protein lysates extracted from p-AKT substrate antibody immunoprecipitation assay. Left lane: control hepatocytes; right lane: *Pten*^{-/-} hepatocytes. The arrows indicate the bands subjected to LC/MS/MS analyses: *a*: leucine-rich PPR motif-containing protein; *b*: monofunctional C1-tetrahydrofolate synthase, lon protease homolog, NAD(P)H transhydrogenase; *c*: aconitase; *d*: glutamate dehydrogenase 1, aldehyde dehydrogenase, ATP synthase subunit α, ATP synthase subunit β (see Table 1). (B) ATP synthase activity of control- and *Pten*^{-/-} hepatocytes (assay conditions as described in the Materials and Methods section). **P < 0.01. (C) Structural features of ATP synthase α protein sequence. Triangles indicate ATP synthase α-β interacting interface. A reverse substrate consensus sequence for AKT substrate is identified from residue 166–171.

Fig. 11. Activation of AKT alters the respiration complexes

(A) Mitochondrial proteins (1mg/ml) were immunoprecipitated with anti-phospho-AKT-substrate antibody (PAS, lane 1 and 2, control and *Pten*^{-/-}) and anti-AKT antibody (lane 3 and 4, control and *Pten*^{-/-}) followed by SDS-PAGE electrophoresis. The membrane was incubated with MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (MS604) containing anti-Complex I subunit NDUFB8 (CI-20), anti-Complex II subunit 30kDa (CII-30), anti-Complex III subunit Core 2 (CIII-core2), anti-Complex IV subunit I (CIV-I), and anti-ATP synthase subunit α (CV- α). Mitochondrial proteins were loaded as control (lane 5 and 6, control and *Pten*^{-/-}). (B) Expression of mitochondrial complexes in total cell lysates. Western blots of mitochondrial complexes in control- and *Pten*^{-/-} immortalized hepatocytes. Mitochondria were isolated from control- and *Pten*^{-/-} immortalized hepatocytes. 20 μg mitochondrial proteins were loaded in each well. The membrane was incubated with MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (MS604); Top, boiled samples; bottom, unboiled samples. (C) Measurement of complexes I, II, and IV activities in mitochondria from control- and *Pten*^{-/-} immortalized hepatocytes. (●) control hepatocytes; (●) *Pten*^{-/-} hepatocytes. ** $P < 0.01$.

Table 1

LC/MS/MS analyses. Xc and Δcn values were calculated using TurboSequest software. Expect scores below 0.05 were considered significant.

	Xc	Δcn	Expect scores
<i>Leucine-rich PPR motif-containing protein</i>			
K.DESSDNFGSFFLR.H	3.82	0.49	1.07×10^{-9}
R.SCGSLLPELSLAER.T	3.6	0.52	1.33×10^{-4}
<i>Monofunctional C1-tetrahydrofolate synthase</i>			
K.LIAEESVSLAAALR.I	3.98	0.49	2.4×10^{-5}
K.YVLVAGITPTPLGEGK.S	4.28	0.52	7.8×10^{-5}
R.FQFLYDVQLPIVEK.I	3.84	0.44	7.50×10^{-11}
<i>Lon protease homolog</i>			
K.AQLSAAVLTLLIK.Q	4.82	0.53	2.2×10^{-6}
K.HVMDVVDEELSK.L	4.07	0.44	1.85×10^{-5}
K.LALLDNHSSEFNVTR.N	4.42	0.61	5.43×10^{-8}
<i>NAD(P)H transhydrogenase</i>			
K.ILIVGGGVAGLASAGAAK.S	4.19	0.53	2.9×10^{-5}
<i>Aconitase</i>			
K.IVYGHLDLDDPANQEIER.G	4.69	0.55	2.2×10^{-10}
K.LTGSLSGWTSPK.D	3.47	0.56	1.9×10^{-5}
K.QGLLPLTFADPSDYNK.I	3.2	0.55	2.0×10^{-5}
<i>ATP synthase subunit α</i>			
K.TSIAIDTHINQK.R	3.75	0.42	2.83×10^{-6}
R.ILGADTSVDLEETGR.V	4.83	0.64	1.03×10^{-12}
R.NVQAEEMVEFSSGLK.G	3.8	0.57	1.68×10^{-7}
R.TGAIVDVPVGEELLGR.V	4.48	0.57	1.22×10^{-9}
<i>ATP synthase subunit β</i>			
R.FTQAGSEVSALLGR.I	4.23	0.62	6.59×10^{-8}
R.IMNVIGEPIDER.G	4.14	0.37	9.76×10^{-5}
R.IPSAVGYQPTLATDMGTMQER.I	4.69	0.65	4.59×10^{-11}

Table 2

Sequence alignment for ATP synthase subunits

Subunit α sequence T/SxxRxR *		
166–171	kgpig S kt R r R vglkapgii	mouse
166–171	kgpv g S k i R r R vglkap	rat
166–171	kgpig S kt R r R vglkapg	human
247–252	valmg E yf R d R gedaliiy	<i>E. coli</i>
Subunit β sequence T/SxxRxR		
453–458	seedkl T vs R a R kqrfl	mouse
399–404	seedkl T vs R a R kqrfl	rat
453–458	seedkl T vs R a R kqrfl	human
390–406	seedkl V va R a R kqrfl	<i>E. coli</i>

* Reserved sequence of AKT substrate consensus sites