Lactate promotes resistance to glucose starvation via upregulation of Bcl-2 mediated by mTOR activation

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Abstract. Solid tumors grow faster and need more glucose than normal tissue; however, due to poor angiogenesis and excessive growth, tumors remote from blood vessels are always under glucose starvation. Even so, cancer cells remain alive *in vivo*. Thus, making cancer cells sensitive to glucose depletion may potentially provide an effective strategy for cancer intervention. Tumors that obtain sufficient glucose generate a large amount of lactate. Therefore, we proposed that lactate, a tumor microenvironment factor, may allow cancer cells to develop resistance to glucose starvation-induced death. We cultured cancer cells in no-glucose medium and added lactate to the medium. During the experiment, lactate helped cancer cells to escape from glucose starvation-induced cell death, without using lactate as an energy substrate, resulting in activation of Akt through PI3K. Akt activation plays a central role in cell growth through the activation of mammalian target of rapamycin (mTOR). Alteration of the PI3K/Akt/mTOR signaling pathway by inhibiting apoptosis induced specific upregulation of B-cell lymphoma 2 (Bcl-2) through translational control. In conclusion, this study showed that lactate rescues cancer cells from glucose starvation-induced cell death through regulation of the PI3K/Akt/mTOR/Bcl-2 signaling pathway. These data suggest that lactate is an important determinant of the sensitivity of tumors to glucose starvation, and reducing lactate or inhibiting the PI3K/Akt/mTOR/Bcl-2 signaling pathway may influence the response of cancers to glucose starvation.

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Introduction

The essential characteristic of tumors is rapid growth; therefore, tumors need to generate a large number of new blood vessels to provide nutrients. Nonetheless, compared with normal tissue, tumor angiogenesis is disarranged and functions poorly (1). The rapid tumor growth and the physiological characteristics of the tumor vasculature mean that the rate of formation of blood vessels providing the energy substances is unable to catch up with the rate of increase in tumor volume, such that many tumors exist in a low glucose environment. The concentration of glucose in colon and stomach tumor tissue is only 0.12 and 0.4 mM, respectively (2). Tumors can survive in harsh environments, such as poor glucose and oxygen depletion (3). In the case of glucose deficiency, tumors *in vivo* adjust their own state to adapt to the environment and obtain more nutrition. By contrast, lack of glucose in cultured tumor cells *in vitro* does not support the survival of tumor cells. Therefore, to explore how tumors survive glucose depletion *in vivo* is important.

Cancer cells preferentially use the glycolysis pathways for energy generation, even in the presence of oxygen, the so called 'aerobic glycolysis', as first proposed by Warburg (4). Glycolysis is far less efficient than oxidative phosphorylation in terms of ATP generation; therefore, cancer cells exhibit abnormally high glycolytic rates to maintain energy homeostasis (5). Such dysregulated metabolism in cancer cells also leads to the accumulation of the metabolic product of glycolysis, lactic acid, in solid tumors. Many measurements have been made to determine the level of tumor lactate and significant variations have been found, with the average ranging from 7 to 10 mM/g and a maximum of up to 25.9 mM/g (6,7). In contrast to tumor hypoxia, tumor glycolysis and lactate biology have received little scientific attention for many years. However, findings concerning the overexpression of glycolysis-related genes in 70% of all human cancers worldwide and the exploitation of increased glucose uptake of cancer cells for tumor diagnostics by positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (FDG), have contributed to the topic experiencing a renaissance. This may lead to improvement of cancer diagnosis and therapeutic follow-up in a clinical setting.

As an important part of the tumor microenvironment, studies of lactate were justified for the following reasons.

Extracellular lactate inhibits the differentiation of monocytes to dendritic cells (DCs) and inactivates cytokine release from DCs (8) and cytotoxic T cells (9), the key players in antitumoral response. The addition of exogenous lactate led to a concentration-dependent increase in random migration of various cancer cell lines (10). Lactate concentrations are positively correlated with radioresistance (11). This reprogramming is necessary for the growth and survival of tumors in stress conditions (12).

In the present study, we found that lactate could rescue cancer cells from glucose starvation-induced death. Furthermore, we explored the mechanism of the role of lactic acid in the process of tumor adaptation to glucose deficiency. We found that lactate rescues cancer cells from glucose starvation-induced cell death by regulating the Akt/mammalian target of rapamycin (mTOR)/B-cell lymphoma 2 (Bcl-2) signaling pathway. These data suggest that lactate is an important determinant of the sensitivity of tumors to glucose starvation, and reducing lactate or inhibiting the Akt/mTOR/Bcl-2 signaling pathway may influence the response of cancers to glucose starvation.

Materials and methods

Cell lines and cell culture. A549, H1299, PC3, DU145 and U87-MG cell lines were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. A549 and U87-MG were cultured in complete Dulbecco's modified Eagle's medium (DMEM, cat. no. 12430-054), with 10% fetal bovine serum (cat. no. 10100-147) (both from Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. DU145 and H1299 were cultured in RPMI-1640 medium (cat. no. 11875-093; Gibco) supplemented as above. PC3 cells were cultured in F-12 medium (cat. no. 21700-075; Gibco) supplemented as above. The glucose concentration in the medium was 25 mM, unless otherwise stated. For the glucose-starvation experiment, we mixed no-glucose DMEM (cat. no. 11966-025) or RPMI-1640 (cat. no. 11879-020) (both from Gibco) with the complete medium mentioned above in a certain proportion to make the glucose concentration 5 mM. We added sodium L-lactate (cat. no. 71718) or L-lactic acid (cat. no. L1750) (both from Sigma-Aldrich, USA) or HCl into the medium to create different culture environments with different lactate concentrations and pHs.

Cell viability assay. Cells (10^4/well) were seeded in 24-well plates in complete medium with 25 mM glucose, 24 h before the experiment. The following day we changed the medium to one containing no glucose. Meanwhile, we added a different concentration of lactate into the medium at the start of the experiment. From day 2 to 12, we counted the live cells every 2 days; 3 wells/day for each culture environment.

Transfection. A small interfering RNA (siRNA) targeting Bcl-2 (cat. no. GS596; Qiagen, Germany) was transfected into cells to block its function. We used Lipofectamine 2000 (cat. no. 11668019; Invitrogen, USA) as the transfection reagent and a negative control siRNA (cat. no. SI03650318; Qiagen). Forty-eight hours after transfection, we collected the mRNA and protein of the transfected cells. To assess knockdown efficiency, we analyzed the mRNA level using real-time PCR (LightCycler 480; Roche, Switzerland) and the level of protein by western blotting (Mini-PROTEAN® 165-8004; Bio-Rad, USA). Both values were normalized to the expression of β-actin. Experiments were performed between 24 and 72 h after transfection.

Analysis of cell metabolism using a Seahorse Bioscience XF24 instrument. The oxygen consumption rate (OCR) measurements of cells were performed using a Seahorse Bioscience XF24 instrument (Seahorse Bioscience, Billerica, MD, USA). Before running the experiment, the growth medium was removed and the cells were washed with PBS containing Ca^{2+}/Mg^{2+} (pH 7.4), which was then aspirated and replaced with 700 ml of reduced serum (RS) buffer [$CaCl_2$ (1.8 mM), $MgCl_2$ (0.6 mM), KH_2PO_4 (0.5 mM), KCl (5.33 mM), Na_2HPO_4 (0.5 mM), NaCl (130 mM), glucose (5.6 mM)], glutamax, minimum essential medium (MEM) amino acid solution, MEM nonessential amino acids, MEM vitamin solution, penicillin/streptomycin, 1% bovine serum albumin (BSA, factor V fatty acid free), 1% FBS and insulin (100 nM). All components, except FBS and insulin, were combined before filter sterilization. Following the addition of FBS and insulin (usually 24-48 h pre-experiment), the RS buffer was warmed to 37˚C and the pH adjusted to 7.4. The template for testing was set up as follows. Measurements were performed every 5 min, repeating metabolic measurements 3-4 times per condition for statistical analyses. The 5-min cycle included a 2-min mix period, a 1-min wait and a 2-min measuring time. After measurements of baseline activity, oligomycin (an ATP coupler) was injected, rates were measured, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (an electron transport chain accelerator) was injected, and finally rotenone and antimycin (mitochondrial inhibitors) were injected, followed by a final rate measurement.

Measurement of extracellular lactate levels. Cells were seeded onto plates and allowed to grow in medium with 25 mM glucose for attachment overnight. The next day, we replaced the medium with fresh medium with 0 mM glucose at time zero. From time zero, every 24 h, we collected the medium samples and counted the number of cells in the same plate. The concentrations of lactate in the medium samples were measured with lactate reagent (cat. no. P0000024; CMA Microdialysis, Sweden). The results were normalized to the number of the cells in each sample.

Measurement of glucose starvation-induced apoptosis. Cells were placed in 6-well plates and incubated for 24 h before the experiment. Cells in the control group were cultured in complete medium containing 25 mM glucose. Cells in the treated group were cultured in complete medium containing 0 mM glucose with different lactate contents. After 48 h of treatment, cells were trypsinized, centrifuged and resuspended in binding buffer with Annexin V-FITC and propidium iodide (PI) from the Dead Cell Apoptosis Kit #2 (cat. no. V13241; Life Technologies, USA). Stained cells were incubated for 15 min at room temperature in the dark. Flow cytometry was used to analyze the stained cells, measuring the fluorescence emission at 530 and 575 nm using 488 nm excitation.

Western blotting and antibodies. Treated cells were lysed using RIPA lysis buffer with 1% phenylmethanesulfonyl fluoride (PMSF). Cell lysates were separated through a 10% SDS gel and blotted onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in PBST at room temperature for 1 h and incubated separately with primary antibodies against Akt (cat. no. 4685), phospho-Akt (Thr308, cat. no. 13038) (both from Cell Signaling Technology, USA), phospho-Akt (Ser473, cat. no. ab66138), PTEN (cat. no. ab32199), Mcl-1 (cat. no. ab114016), Bcl-2 (cat. no. ab117115), Bcl-xL (cat. no. ab32370), phospho-mTOR (Ser2448, cat. no. ab84400) (all from Abcam, UK) or β-actin (cat. no. A1978; Sigma-Aldrich) overnight at 4˚C. The next day, the membranes were washed 3 times with PBST and incubated with secondary antibodies for 1 h at room temperature. After washing the membranes 3 times with PBST, the membranes were scanned using an Odyssey Infrared Imaging System (Li-COR Biosciences, USA).

Reagents. LY294002, perifosine and rapamycin were purchased from Selleck Chemicals, USA (cat. nos. S1105, S1037 and S1039). Insulin and resveratrol were purchased from Sigma-Aldrich (cat. no. R5010). Insulin-like growth factor-1 (IGF-1) was purchased from Invitrogen (cat. no. PHG0071).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5.0 and Instat 3.1 packages (GraphPad Software, Inc., San Diego, CA, USA). The results are expressed as the means ± standard error (SE). Statistical differences between the groups were compared using t-tests. P-values <0.05 were considered to indicate statistically significant results.

Results

Lactate rescues cancer cells from glucose starvation-induced cell death. To determine the effect of lactate on glucose starvation, we exposed A549 cells to different culture conditions: complete DMEM containing different sodium lactate concentrations and without glucose. We found that in glucose-free conditions, adding sodium lactate into the medium effectively prolonged the survival times of the A549 cell line (Fig. 1A). At low doses, cell proliferation and survival time increased with the increment of sodium lactate concentration. The survival time of A549 cells in the presence of 20 mM sodium lactate was longer than that of any other concentrations of sodium lactate. Indeed, A549 cells grown with sodium lactate in glucose-free conditions survived for more than 2 weeks; even 1 month later there were still viable cells. At sodium lactate concentrations >20 mM, cell proliferation and survival time decreased. Without sodium lactate, in A549 cells onset of cell death started in 1-2 days and all the cells died after 4 days. However, high concentrations of sodium lactate (such as 80 mM) caused immediate death of A549 cells (Fig. 1B).

We also confirmed the above phenomena in other types of tumor cell lines. We cultured four human cancer cell lines derived from the brain (U87-MG), prostate (PC3 and DU145), and lung (H1299) in no-glucose cultivation conditions with various lactate concentrations. The addition of sodium lactate (20 mM) effectively prolonged the survival time of all 5 types of tumor cells. We then compared the proliferation and survival time between all 5 types of cancer cells in the presence of sodium lactate. We found that DU145, A549 and H1299 cell proliferation activity was stronger than PC3 and U87-MG cells (Fig. 1C), and their survival times were longer than those of the PC3 and U87-MG cell lines (Fig. 1D).

Some studies have reported that tumor cells have the ability to take up lactate and utilize it as an energy source via oxidative phosphorylation. Determination of dissolved oxygen in the culture of cells monitored in the Seahorse Bioscience XF24 instrument showed that, upon addition of lactate, oxidative phosphorylation levels did not change significantly (Fig. 1E). In addition, we examined the lactate concentrations in the cell culture; the extracellular lactate concentrations did not change significantly over the course of the experiment (Fig. 1F). These results suggest that under deprivation of glucose, lactate was not used as a substrate for energy metabolism.

Cell secretion of lactic acid would affect the value of pH in the extracellular environment. To confirm whether the acidic environment plays a role in tumor cell survival in a glucose-free environment, we cultured A549 cells separately in the following 4 types of environment: no glucose, no glucose + 20 mM sodium lactate (pH 7.4), no glucose + 20 mM lactic acid (pH 6.8), no glucose + 20 mM hydrochloric acid (pH 6.8). After 72 h of culture, we found that adding hydrochloric acid to the tumor cells in glucose-free environment had no significant effect on survival, adding lactic acid had some effect, and adding lactate had the most significant effect (Fig. 1G). We concluded that tumor cell survival in a glucose-free environment was mainly a function of lactate itself, and had little to do with the acidic environment.

Effect of activation of Akt signaling on the role of lactate in tumor cell survival in glucose-free conditions. In the above experiments, we found that sodium lactate had different effects on the survival of the different cell lines in glucose-free conditions. Accordingly, we divided the five cancer cell lines into 2 groups: a lactate-sensitive group, including DU145, A549 and H1299; and a lactate-insensitive group, including PC3 and U87-MG. To explore why lactate had different effects on the survival of the 2 groups of cells in glucose-free conditions, we compared the genetic background of the 2 groups of cells. We found that the 2 lactate insensitive cell lines, PC3 and U87-MG, had lost PTEN activity (phosphatase and tensin homolog deleted on chromosome 10) (Fig. 2A). PTEN is a tumor-suppressor gene with phosphatase activity, which is involved in the negative regulation of the Akt signaling pathway, where it blocks the activation of Akt and its downstream effector molecules (13). The Akt signaling pathway plays an important role in cell proliferation and survival (14). Therefore, to confirm the existence of activation of the Akt signaling pathway in PC3 and U87-MG cell lines with PTEN deletion, we examined the phosphorylation status of Akt in the 2 cell lines (Fig. 2B). The results showed that, compared with the lactate-sensitive group, Akt phosphorylation in the lactate-insensitive group increased, which would result in Akt signaling pathway activation. We also compared the reactions of the 2 groups of cells to insulin, an activator of the Akt signaling pathway (Fig. 2C). Insulin caused a dose-dependent

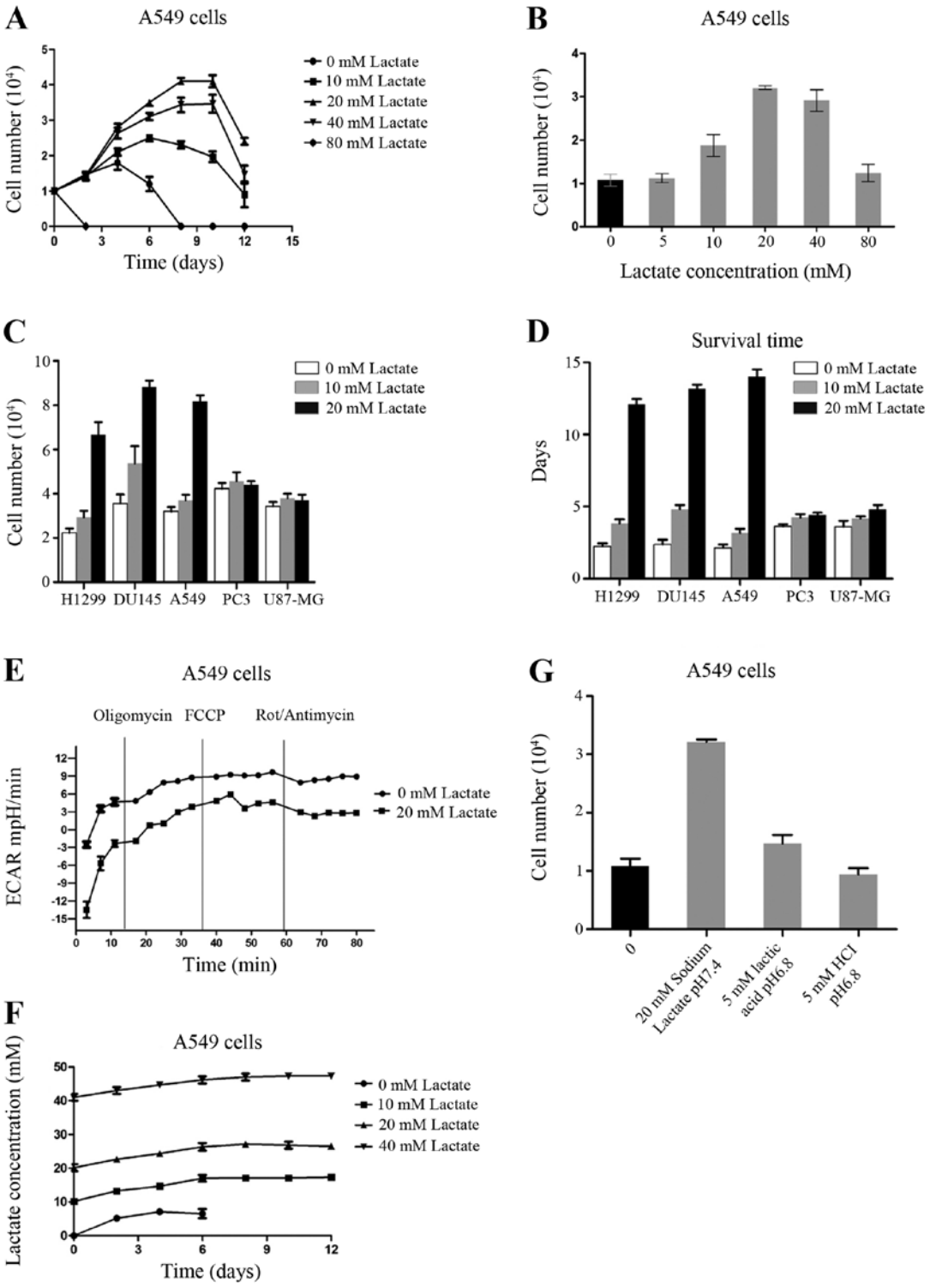

Figure 1. Lactate promotes cancer cell resistance to glucose starvation. (A) Cell growth curves of A549 cells, which were cultured in glucose-free medium with different concentrations of lactate. (B) The numbers of A549 cells were counted 3 days after culture in glucose-free medium with different concentrations of lactate. (C) The numbers of H1299, DU145, A549, PC3 and U87-MG cells counted 3 days after culture in glucose-free medium with different concentrations of lactate. (D) Survival of H1299, DU145, A549, PC3 and U87-MG cells after culture in glucose-free medium with different concentrations of lactate. (E) The oxygen consumption rate (OCR) of A549 cells with or without lactate was measured at baseline and continuously throughout the experimental period and in the presence of the indicated drugs: oligomycin (1 μg/ml), carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (1 μM), rotenone (1 μM) plus antimycin A (1 μM). (F) The lactate concentration in the medium was detected under the same experimental conditions as in A. (G) The numbers of A549 cells were counted 3 days after culture in different environments: no glucose, no glucose + 20 mM sodium lactate (pH 7.4), no glucose + 5 mM lactic acid (pH 6.8), and no glucose + 5 mM hydrochloric acid (pH 6.8). Data are mean ± standart error (SE), n=3.

increase in cell numbers in the cells of the lactate-sensitive group. By contrast, the growth of the cells in the lactate-insensitive group was not affected by insulin. The above results suggest that the state of the Akt signaling pathway was

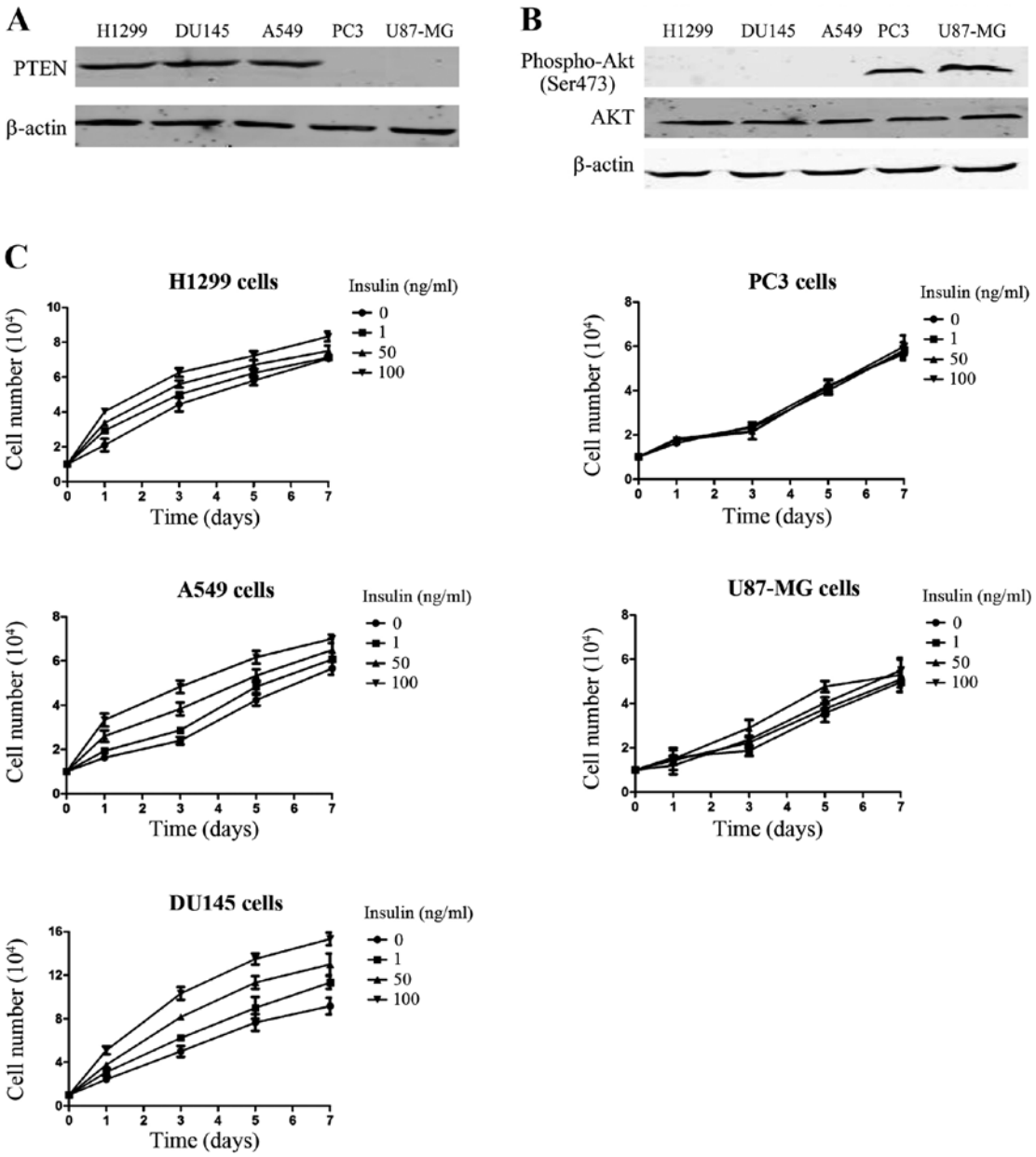

Figure 2. Activation of Akt signaling correlates with the role of lactate in glucose starvation. (A) Protein expression of PTEN in five cell lines (H1299, DU145, A549, PC3 and U87-MG) was examined by western blotting. (B) Protein expression of phospho-S473 Akt and total Akt in five cell lines (H1299, DU145, A549, PC3 and U87-MG) was examined by western blotting. β-actin was used as the normalization control. (C) Proliferation curves of cancer cells from five cell lines cultured in the presence of increasing concentrations of insulin. Data are mean ± standard error (SE), n=3.

a key factor that distinguished the role of lactate in cancer cell proliferation and survival time in glucose-free conditions.

Lactate induces Akt phosphorylation through PI3K. To analyze Akt signaling induced by lactate, A549 cells were cultured overnight in DMEM without serum, and the cells were stimulated with sodium lactate for 30 min. Lysates of these cells were analyzed by western blotting, and Akt activation was measured by detection of Thr308 and Ser473 phosphorylated forms of Akt. We used insulin to stimulate the serum-deprived cells as a positive control for the activation of Akt. Under this environment, we observed that Akt Thr308 and Ser473 phosphorylation increased in the presence of lactate to a similar extent to that obtained with insulin (Fig. 3A and C). We then used perifosine (an inhibitor of Akt) to suppress the activation of Akt. We found that Akt Thr308 and Ser473 phosphorylation by lactate or insulin were suppressed by perifosine (Fig. 3C). We obtained the same result when we used different cell lines, such as H1299 and DU145, indicating that the differences in Akt phosphorylation were not specific to the A549 cell line (Fig. 3B). Time course experiments showed that Akt phosphorylation at Thr308 and Ser473 phosphorylation sites were both rapid (1-5 min after incubation with lactate), with maximum levels being achieved between 10 and 30 min (Fig. 3D). To estimate whether lactate induced Akt phosphorylation downstream of PI3K, we used LY294002 (an inhibitor of PI3K). Akt Thr308 and Ser473 phosphorylation in the presence of lactate were substantially

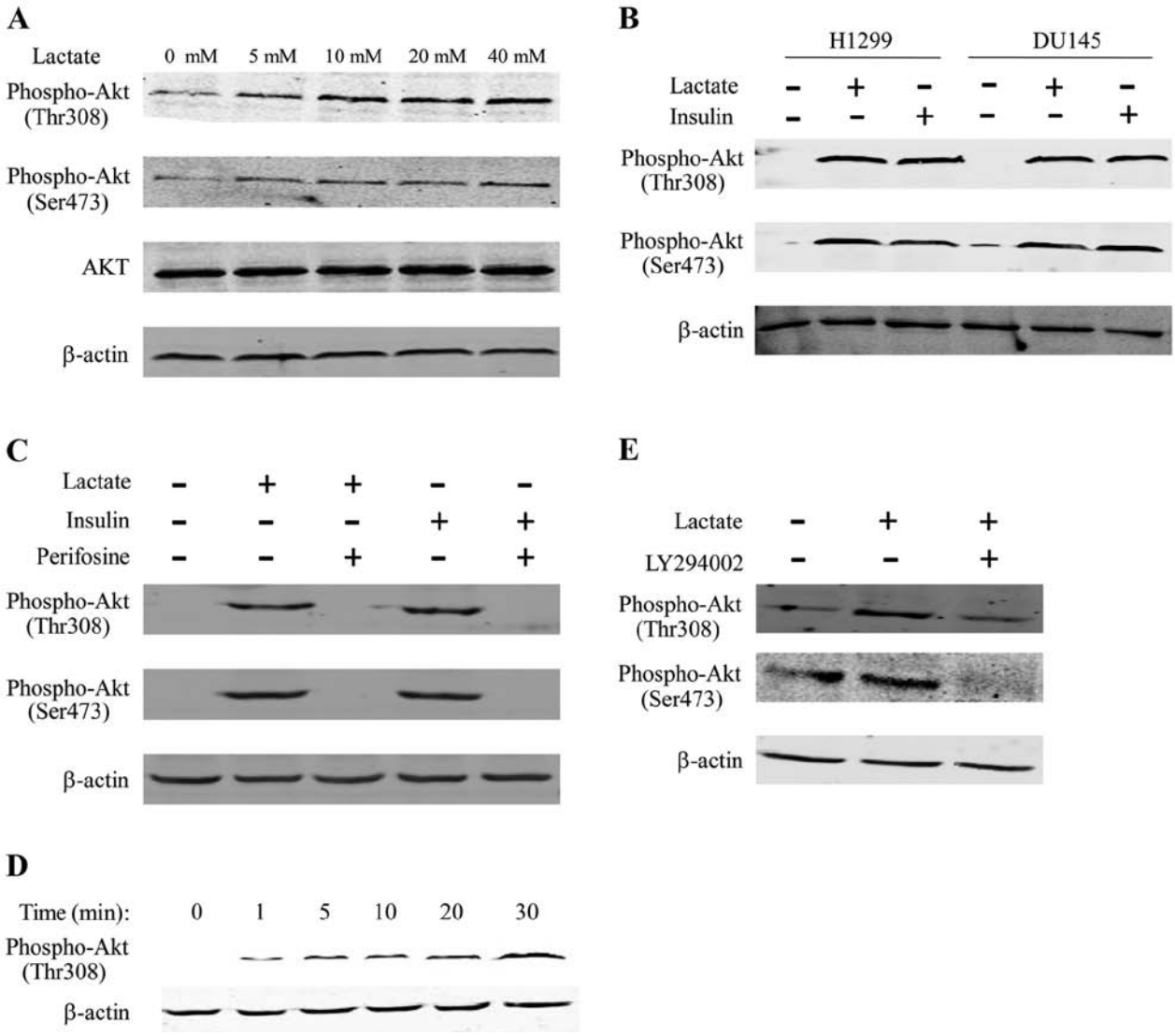

Figure 3. Akt is phosphorylated at Thr308 and Ser473 in response to lactate, mediated by PI3K. (A) Protein expression of phospho-Thr308 Akt, phospho- Ser473 Akt and total Akt in the A549 cell line with different concentrations of lactate was examined by western blotting. (B) Protein expression of phospho-Thr308 Akt, phospho-Ser473 Akt in the H1299 and DU145 cell lines with lactate (20 mM) or insulin (100 nM) was examined by western blotting. (C) Protein expression of phospho-Thr308 Akt, phospho-Ser473 Akt in the A549 cell line treated with lactate (20 mM) or insulin (100 nM) or perifosine (1 μM) was examined by western blotting. (D) Protein expression of phospho-Thr308 Akt in the A549 cell line stimulated with lactate (20 mM) for 30 min was examined by western blotting. (E) Protein expression of phospho-Thr308 Akt, phospho-Ser473 Akt in the A549 cell line treated with lactate (20 mM) or LY294002 (5 μM) was examined by western blotting. β-actin was used as the normalization control.

reduced in the presence of LY294002 (Fig. 3E). These results suggest that lactate induced the activation of Akt significantly and rapidly through Thr308 and Ser473 phosphorylation mediated by PI3K.

Lactate activates the Akt/mTOR/Bcl-2 signaling pathway to modulate the apoptotic response of cancer cells to glucose starvation. Akt plays an important role in cell apoptosis and survival in response to extracellular stimuli, such as insulin or growth factors. Our previous experiments showed that the state of the PI3K/Akt signaling pathway was a key factor to distinguish the effect of lactate on cancer cell proliferation and survival in glucose-free conditions, where lactate induces the activation of Akt by Thr308 and Ser473 phosphorylation. We hypothesized that lactate helps tumor cells to survive by activating Akt in glucose-free conditions. To test the hypothesis, we cultured A549 cells with perifosine, an Akt inhibitor. We found that treatment with perifosine inhibited the cell survival induced by lactate in glucose-free conditions. In addition, treatment with an Akt activator, IGF-1, increased A549 cell survival in glucose-free conditions, but less effectively compared with lactate (Fig. 4A). These results showed that the lactate increased tumor cell survival in glucose free conditions through Akt activation.

Many studies have demonstrated that under the metabolic stress of no glucose, tumor cells undergo significant apoptosis. Thus, we investigated metabolic stress resistance to apoptosis in tumor cells incubated with lactate (Fig. 4B). We cultured A549 cells in high glucose (25 mM glucose), glucose-free and glucose-free with added lactate conditions. Twenty-four hours later, cells were trypsinized, centrifuged and resuspended in binding buffer with Annexin V-FITC and PI. The stained cells

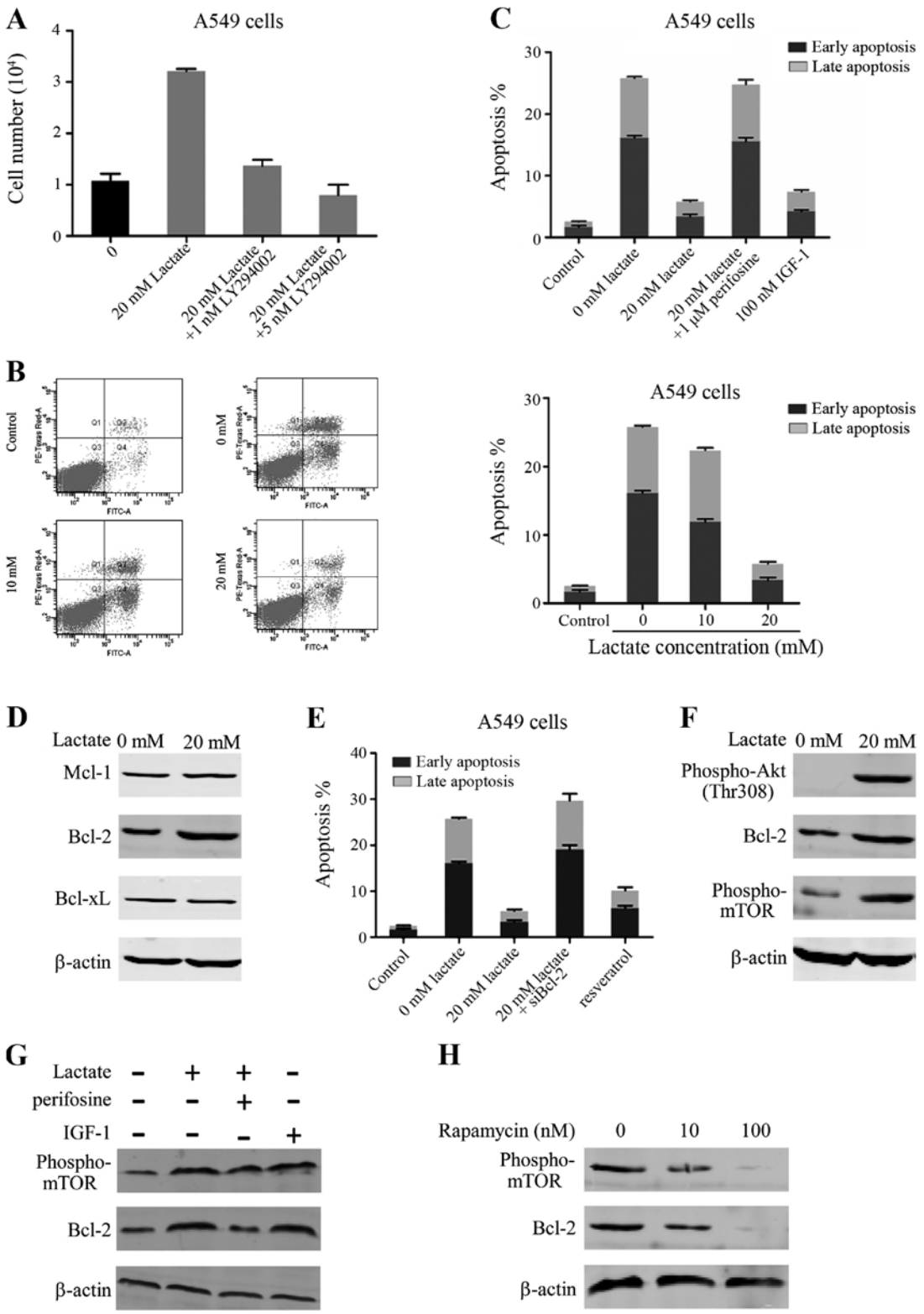

Figure 4. Reduced apoptosis by lactate during glucose starvation is mediated through the activation of the PI3K/Akt/mammalian target of rapamycin (mTOR)/B-cell lymphoma (Bcl-2) signaling pathway. (A) The numbers of A549 cells were counted 3 days after culture in glucose-free medium treated with lactate (20 mM) or lactate (20 mM) + LY294002 (1 nM) or lactate (20 mM) + LY294002 (5 nM). (B) A549 cells were cultured in 25 mM glucose medium or no glucose medium with lactate 48 h before staining with Annexin V-FITC and propidium iodide (PI). Flow cytometry to measure the fluorescence intensity at 530 and 575 nm, using 488 nm excitation, allowed us to calculate the early and late apoptosis rates of the cells. (C) The early and late apoptosis rates of A549 cells were calculated after they were cultured in 25 mM glucose medium, no glucose medium with lactate (20 mM), lactate (20 mM) + perifosine (1 μM) or IGF-1 (100 nM) for 48 h. (D) Protein expression of Mcl-1, Bcl-2 and Bcl-xL in the A549 cell line treated with or without lactate (20 mM) in glucose-free medium was examined by western blotting. (E) The early and late apoptosis rates of A549 cells were calculated after they were cultured in 25 mM glucose medium, no glucose medium with lactate (20 mM) + control small interfering RNA (siRNA), lactate (20 mM) + siBcl-2 or resveratrol (100 μM) for 48 h. (F) Protein expression of phospho-Thr308 Akt, Bcl-2 and phospho-Ser2448 mTOR in the A549 cell line treated with or without lactate (20 mM) in glucose-free medium was examined by western blotting. (G) Protein expression of phospho-Ser2448 mTOR and Bcl-2 in the A549 cell line treated with lactate (20 mM), lactate (20 mM) + perifosine (1 μM), or IGF-1 (100 nM) was examined by western blotting. (H) Protein expression of phospho-Ser2448 mTOR and Bcl-2 in the A549 cell line treated with rapamycin was examined by western blotting. β-actin was used as the normalization control.

were analyzed by flow cytometry. We found that compared with high glucose conditions, there were many apoptotic cells in the glucose-free environment. There was an ~10.1-fold ($P<0.001$) increase in early apoptosis and an ~12-fold ($P<0.001$) increase in late apoptosis in the population of cells without glucose compared to the cells with high glucose. The apoptosis rate in the culture with added lactate culture was greatly reduced, and was close to that under the high glucose cultivation. The above results showed that under glucose-free conditions, a large number of A549 underwent apoptosis, and the addition of lactate could prevent this apoptosis, the mechanism of which will be further explored below.

We checked the apoptosis rate of A549 cells and then added perifosine or IGF-1. We found that treatment with perifosine prevented the decrease in apoptosis induced by lactate in glucose-free conditions, and that IGF-1 reduced the cell apoptosis caused by no glucose (Fig. 4C). We concluded that lactate prevented apoptosis in glucose-free conditions through Akt activation.

Several studies have pointed out that the anti-apoptotic Bcl-2 family is pivotal for cell survival under metabolic stress. We found lactate can reduce tumor cell apoptosis caused by the lack of glucose. We, therefore, examined whether lactate affected the expression levels of anti-apoptotic Bcl-2 family proteins such as Mcl-1, Bcl-2 and Bcl-xL. A549 cells were cultured with added lactate in glucose-free conditions. After 48 h, lysates of these cells were analyzed by western blotting. Treatment with lactate increased Bcl-2 levels, while Mcl-1 or Bcl-xL levels were not affected by treatment with lactate (Fig. 4D). To confirm whether Bcl-2 plays a major role in cell survival, we used a Bcl-2 targeting siRNA to lower its protein expression. The results showed that treatment with siBcl-2 prevented resistance to apoptosis induced by lactate in glucose-free conditions. Furthermore, we observed that resveratrol, a Bcl-2 activator, reduced the apoptosis of A549 cells in glucose-free conditions, similarly to lactate (Fig. 4E). These findings revealed that the upregulation of Bcl-2 by lactate is important for tumor cell resistance to apoptosis in glucose-free conditions.

Akt plays a core role in promoting cell survival, through activation of anti-apoptotic substances. Therefore, we aimed to confirm whether Bcl-2 upregulation is mediated via activation of the Akt/mTOR signaling pathway upon treatment with lactate in glucose-free conditions. First, we found that in A549 cells treated with lactate, Akt was activated and the expression of the Bcl-2 protein was increased, accompanied by mTOR activation (Fig. 4F). To demonstrate that the increase in the expression levels of Bcl-2 and mTOR were dependent on the activation of Akt, we added perifosine and found that the expression levels of Bcl-2 and mTOR were restored to the original levels. Furthermore, the expression levels of Bcl-2 and mTOR were upregulated in the presence of IGF-1 (Fig. 4G). Addition of rapamycin, an mTOR inhibitor, blocked the increased expression of Bcl-2 (Fig. 4H). These results demonstrated that Akt activation and mTOR upregulation by lactate in glucose-free conditions led to the upregulation of Bcl-2.

Taking all the above results into consideration, we conclude that lactate, through activation of Akt by phosphorylation mediated by PI3K, activates mTOR and further increases the expression of anti-apoptotic protein Bcl-2, to help tumor cells resist apoptosis caused by glucose starvation.

Discussion

Reprogramming energy metabolism is a hallmark of cancer (5). Warburg (4) first observed that even in cases with an adequate oxygen supply, tumors still preferred to utilize glucose via glycolysis. Compared with oxidative phosphorylation, glycolysis is a low efficiency method of energy production; therefore, compared with normal tissue, tumors often require more glucose. The clinical diagnosis of cancer by PET with a radiolabeled analog of glucose (FDG) as a reporter, a widely used method, is possible as tumor cells have increased glucose uptake (15). These changes in tumor metabolic patterns, increased glucose uptake and glycolysis as the main production method, eventually lead to the accumulation of lactate, the end product of glycolysis, in tumors. Several studies have reported increased lactate levels within tumors, which reflects the high metastasis rate and poor prognosis in human cervical cancers (16), human head and neck cancers (17), human rectal adenocarcinomas (18), human hepatocellular carcinoma (19) and non-small cell lung cancers (20). Some studies have suggested that lactate could be used as an energy source by oxidative phosphorylation to generate ATP (21,22). Lactate can also be used as a signaling molecule in tumor cells (23). Lactate can produce the promotion of VEGF in wound healing (24), and lactate is also sufficient to instigate signals for angiogenesis (25).

In solid tumors, along with the rapid growth of the tumor, the development of blood vessels within is incomplete, which leads to certain areas of the tumor to suffer glucose deficit. In blood-rich regions, aerobic glycolysis consumes a large amount of glucose to produce lactate, whereas the lactate in the blood-poor regions of tumor cells plays an important role. In the present study, in the absence of glucose, added lactate in culture significantly prolonged the survival of A549 cells in a concentration-dependent manner. This result was consistent with those of the Wu *et al* (26). Subsequent experiments in different cell lines confirmed the role of lactate. Some studies reported that lactate could be used as an energy substrate to produce ATP by tumor cells through oxidative phosphorylation; we determined whether under no glucose conditions, lactate could be used as an energy substrate. The results showed no significant changes in oxygen consumption and or any lactate consumption. Thus, under glucose-free conditions, lactate is not an energy substrate. We also ruled out an acidic environment in glucose-free conditions as having any influence on maintaining tumor cell growth.

Notably, lactate had different effects on different cell lines, allowing cell lines to be divided into sensitive and insensitive lactate groups. We compared the genetic backgrounds of the five cell lines and found that the cell lines insensitive to lactate lacked PTEN function. Insulin-mediated stimulation of growth confirmed the activation of the Akt signaling pathway in the insensitive cell lines. Therefore, to further explore the mechanism of lactate, we focused on the Akt signaling pathway. Akt signaling pathway is an important signaling pathway in tumor cell survival and development. It is activated by upstream signaling molecules, such as growth factors, and is then

further regulated by downstream molecules to participate in the occurrence and development of tumors (14). The activation of the PI3K/Akt pathway could help tumor resistance under dietary deficient conditions (27). Western blotting showed that lactate activated Akt via the rapid phosphorylation at Thr308 and Ser473 mediated by PI3K. Lactate can be used as a signaling molecule in the regulation of certain signaling pathways. For example, lactate was found to upregulate the transcription of 673 genes in L6 cells and was further involved in mitochondrial biogenesis (28). In tumors, lactate, in the presence of oxygen, stimulated the expression of HIF-1α and upregulated various hypoxia-inducible dependent genes (29). Lactate could increase TLR4 signaling and NF-κB pathway-mediated gene transcription in macrophages (30). Lactate increased the level of TGF-β2 in glioma (31). Interestingly, further experiments demonstrated that lactate helped tumor cell survival in glucose-free conditions through activation of Akt by phosphorylation.

Apoptosis tends to occur when cells are under metabolic stress because of lack of glucose. We found significant apoptosis of A549 cells under glucose-free environment conditions, and the addition of lactate prevented this apoptosis. The Bcl-2 family of anti-apoptotic proteins are key regulators of cell apoptosis (32). We found that lactate increased the expression of Bcl-2, and downregulation of Bcl-2 protein expression using an siRNA reduced the resistance to apoptosis induced by lactate in glucose-free conditions.

The PI3K/Akt signaling pathway plays a key role in inhibiting apoptosis, thereby promoting cell proliferation. The activation of Akt can act directly on apoptosis-related proteins to regulate apoptosis. Activation of Akt induces phosphorylation of caspase-9 at the Ser196 site, which inhibits apoptosis (33). The PI3K/Akt signaling pathway can directly or indirectly affect the functions of transcription factors to regulate cell survival. Akt can inhibit the enzyme IκBα that phosphorylates NF-κB, whereas unphosphorylated NF-κB in the nucleus regulates anti-apoptotic gene transcription (34). Akt can prevent the mitochondrial release of cytochrome *c* and apoptosis-inducing factors, contributing to apoptosis resistance (35). In the present study, we confirmed that lactate acts via the PI3K/Akt pathway to regulate Bcl-2, inhibiting apoptosis caused by the lack of glucose.

In conclusion, lactate helps tumor cells to resist apoptosis caused by glucose starvation. Lactate, through PI3K, activates Akt by phosphorylation, which activates mTOR and further increases the expression of anti-apoptotic protein Bcl-2. This study indicates that treatments targeting lactate could more effectively inhibit the survival of tumors.

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References

1. Vaupel P: Tumor microenvironmental physiology and its implications for radiation oncology. Semin Radiat Oncol 14: 198-206, 2004.
2. Hirayama A, Kami K, Sugimoto M, Sugawara M, Tok N, Onozuka H, Kinoshita T, Saito N, Ochiai A, Tomita M, Esumi H and Soga T: Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. Cancer Res 69: 4918-4925, 2009.
3. Hahnfeldt P, Panigrahy D, Folkman J and Hlatky L: Tumor development under angiogenic signaling: a dynamical theory of tumor growth, treatment response, and postvascular dormancy. Cancer Res 59: 4770-4775, 1999.
4. Warburg O: On respiratory impairment in cancer cells. Science 124: 269-270, 1956.
5. Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. Cell 144: 646-674, 2011.
6. Quennet V, Yaromina A, Zips D, Rosner A, Walenta S, Baumann M and Mueller-Klieser W: Tumor lactate content predicts for response to fractionated irradiation of human squamous cell carcinomas in nude mice. Radiother Oncol 81: 130-135, 2006.
7. Brizel DM, Schroeder T, Scher RL, Walenta S, Clough RW, Dewhirst MW and Mueller-Klieser W: Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. Int J Radiat Oncol Biol Phys 51: 349-353, 2001.
8. Gottfried E, Kunz-Schughart LA, Ebner S, Mueller-Klieser W, Hoves S, Andreesen R, Mackensen A and Kreutz M: Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. Blood 107: 2013-2021, 2006.
9. Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, Gottfried E, Schwarz S, Rothe G, Hoves S, Renner K, Timischl B, Mackensen A, Kunz-Schughart L, Andreesen R, Krause SW and Kreutz M: Inhibitory effect of tumor cell-derived lactic acid on human T cells. Blood 109: 3812-3819, 2007.
10. Goetze K, Walenta S, Ksiazkiewicz M, Kunz-Schughart LA and Mueller-Klieser W: Lactate enhances motility of tumor cells and inhibits monocyte migration and cytokine release. Int J Oncol 39: 453-463, 2011.
11. Sattler UG, Meyer SS, Quennet V, Hoerner C, Knoerzer H, Fabian C, Yaromina A, Zips D, Walenta S, Baumann M and Mueller-Klieser W: Glycolytic metabolism and tumour response to fractionated irradiation. Radiother Oncol 94: 102-109, 2010.
12. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, Royer RE, Vander Jagt DL, Semenza GL and Dang CV: Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proc Natl Acad Sci USA 107: 2037-2042, 2010.
13. Sharrard RM and Maitland NJ: Regulation of protein kinase B activity by PTEN and SHIP2 in human prostate-derived cell lines. Cell Signal 19: 129-138, 2007.
14. Osaki M, Oshimura M and Ito H: PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis 9: 667-676, 2004.
15. Gambhir SS, Czernin J, Schwimmer J, Silverman DH, Coleman RE and Phelps ME: A tabulated summary of the FDG PET literature. J Nucl Med 42 (Suppl 5): 1S-93S, 2001.
16. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfør K, Rofstad EK and Mueller-Klieser W: High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. Cancer Res 60: 916-921, 2000.
17. Brizel DM, Schroeder T, Scher RL, Walenta S, Clough RW, Dewhirst MW and Mueller-Klieser W: Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. Int J Radiat Oncol Biol Phys 51: 349-353, 2001.
18. Walenta S, Chau TV, Schroeder T, Lehr HA, Kunz-Schughart LA, Fuerst A and Mueller-Klieser W: Metabolic classification of human rectal adenocarcinomas: a novel guideline for clinical oncologists? J Cancer Res Clin Oncol 129: 321-326, 2003.
19. Sheng SL,Liu JJ, Dai YH, Sun XG, Xiong XP and Huang G: Knockdown of lactate dehydrogenase A suppresses tumor growth and metastasis of human hepatocellular carcinoma. FEBS J 279: 3898-3910, 2012.

20. Yokota H, Guo J, Matoba M, Higashi K, Tonami H and Nagao Y: Lactate, choline, and creatine levels measured by vitro 1H-MRS as prognostic parameters in patients with non-small-cell lung cancer. J Magn Reson Imaging 25: 992-999, 2007.
21. Semenza GL: Tumor metabolism: cancer cells give and take lactate. J Clin Invest 118: 3835-3837, 2008.
22. Bonuccelli G, Tsirigos A, Whitaker-Menezes D, Pavlides S, Pestell RG, Chiavarina B, Frank PG, Flomenberg N, Howell A, Martinez-Outschoorn UE, Sotgia F and Lisanti MP: Ketones and lactate 'fuel' tumor growth and metastasis: Evidence that epithelial cancer cells use oxidative mitochondrial metabolism. Cell Cycle 9: 3506-3514, 2010.
23. Vegran F, Boidot R, Michiels C, Sonveaux P and Feron O: Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-κB/IL-8 pathway that drives tumor angiogenesis. Cancer Res 71: 2550-2560, 2011.
24. Trabold O, Wagner S, Wicke C, Scheuenstuhl H, Hussain MZ, Rosen N, Seremetiev A, Becker HD and Hunt TK: Lactate and oxygen constitute a fundamental regulatory mechanism in wound healing. Wound Repair Regen 11: 504-509, 2003.
25. Hunt TK, Aslam R, Hussain Z and Beckert S: Lactate, with oxygen, incites angiogenesis. Adv Exp Med Biol 614: 73-80, 2008.
26. Wu H, Ding Z, Hu D, Sun F, Dai C, Xie J and Hu X: Central role of lactic acidosis in cancer cell resistance to glucose deprivation-induced cell death. J Pathol 227: 189-199, 2012.
27. Kalaany NY and Sabatini DM: Tumours with PI3K activation are resistant to dietary restriction. Nature 458: 725-731, 2009.
28. Hashimoto T, Hussien R, Oommen S, Gohil K and Brooks GA: Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis. FASEB J 21: 2602-2612, 2007.
29. Lu H, Forbes RA and Verma A: Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem 277: 23111-23115, 2002.
30. Samuvel DJ, Sundararaj KP, Nareika A, Lopes-Virella MF and Huang Y: Lactate boosts TLR4 signaling and NF-kappaB pathwaymediated gene transcription in macrophages via monocarboxylate transporters and MD-2 up-regulation. J Immunol 182: 2476-2484, 2009.
31. Baumann F, Leukel P, Doerfelt A, Beier CP, Dettmer K, Oefner PJ, Kastenberger M, Kreutz M, Nickl-Jockschat T, Bogdahn U, Bosserhoff AK and Hau P: Lactate promotes glioma migration by TGF-beta2-dependent regulation of matrix metalloproteinase-2. Neuro Oncol 11: 368-380, 2009.
32. Cory S and Adams JM: The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2: 647-656, 2002.
33. Song G, Ouyang GL and Bao SD: The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 9: 59-71, 2005.
34. Jeong SJ, Pise-Masison CA, Radonovich MF, Park HU and Brady JN: Activated Akt regulates NF-kappaB activation, p53 inhibition and cell survival in HTLV-1-transformed cells. Oncogene 24: 6719-6728, 2005.
35. Kim EC, Yun BS, Ryoo IJ, Min JK, Won MH, Lee KS, Kim YM, Yoo I and Kwon YG: Complestatin prevents apoptotic cell death: inhibition of a mitochondrial caspase pathway through AKT/PKB activation. Biochem Biophys Res Commun 313: 193-204, 2004.