Capsaicin inhibits glycolysis in esophageal squamous cell carcinoma by regulating hexokinase-2 expression

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Abstract. Capsaicin is a principal component of hot red peppers and chili peppers. Previous studies have reported that capsaicin exhibits antitumor functions in a variety of tumor models. Although various mechanisms underlying the capsaicin-mediated inhibition of tumor growth have been demonstrated, the impact of capsaicin on tumor metabolism has rarely been reported. The present study demonstrated that capsaicin exhibited an inhibitory effect on tumor glycolysis in esophageal squamous cell carcinoma (ESCC) cells. Following treatment with capsaicin, glucose consumption and lactate production in ESCC cells was decreased. Capsaicin resulted in a decrease of hexokinase-2 (HK-2) expression, which is known for its important role in tumor glycolysis. Further investigations demonstrated that phosphatase and tensin homolog (PTEN) expression was increased in ESCC cells treated with capsaicin, and that the RAC-α serine threonine-protein kinase signaling pathway was downregulated. In PTEN-knockdown KYSE150 cells, the decrease in HK-2 and inhibition of glycolysis caused by capsaicin was attenuated, which suggested that the impact of capsaicin on tumor metabolism was associated with its effect on PTEN.

Introduction

Capsaicin, which is a principal component of red peppers and hot chili peppers, has traditionally been used to treat a variety of neuropathic pain conditions, including rheumatoid arthritis, diabetic neuropathy, cluster headaches and herpes zoster (1-3). The anticancer activity of capsaicin has also been identified in various types of tumor. Capsaicin has been demonstrated to exhibit inhibitory activity against tumor growth in human leukemia cells (4), lung cancer (5), colon cancer (6), gastric cancer (7), prostate cancer (8) and hepatocellular carcinoma cells (9). Investigation into the underlying mechanisms has demonstrated that treatment with capsaicin induced tumor cells to undergo cell cycle arrest and apoptosis (10). Previous studies have reported that capsaicin inhibited the translocation of nuclear factor-κB and activator protein-1 (11), and the signal transducer and activator of transcription-3 signaling pathway (12), which were required for cancer development. However, the effect of capsaicin on tumor glycolysis remains unclear.

In mammalian tissues, as a source of cellular energy and precursor carbon source for biosynthesis, glucose is an indispensable metabolite. The majority of tissues metabolize glucose to pyruvate and, in the presence of oxygen, harness the energy within this molecule in the form of ATP via oxidative phosphorylation, in which pyruvate is converted into CO_2. By contrast, tumor tissues exhibit an increase in the less-efficient process of anoxic regeneration of NAD^+, in which pyruvate is converted into lactate even in oxygen-rich conditions; this is termed aerobic glycolysis or the Warburg effect, in order to separate it from the normal glycolysis. Tumor glycolysis supplies energy for tumor rapid growth, and the secretion of lactate provides an appropriate microenvironment for tumor cells to evade apoptosis and metastasize (13). The conversion of glucose to glucose-6-phosphate, an essential and irreversible step in tumor glycolysis, is catalyzed by hexokinases (HK). A total of four different HK isoforms, termed HK-1-4, have been identified (14). HK-1 is ubiquitously expressed, whereas HK-2 is expressed in limited types of tissues. In malignant tumors, particularly in tumors with a highly glycolytic phenotype, HK-2 is overexpressed, whereas HK-1 is expressed to a lesser extent, suggesting a predominant role of HK-2 in the regulation of tumor glycolysis (15). 2-Deoxy-D-glucose, an analogue of glucose, is able to be phosphorylated by HK-2 and not metabolized further; it may be labeled with the positron emitter ^{18}F-fluorodeoxyglucose (^{18}F-FDG) and used for positron emission tomography (PET) scanning to detect cancers in a non-invasive way (16). Numerous clinical studies have demonstrated that the overexpression of HK-2 was associated with poor prognosis in patients with various types of cancer, including pancreatic cancer (17), ovarian cancer (18), hepatocellular carcinoma (19) and esophageal adenocarcinoma (20).

In the present study, the effect of capsaicin on tumor growth and glycolysis in esophageal squamous cell carcinoma

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(ESCC) was investigated. The potential mechanism by which capsaicin may inhibit glycolysis was investigated. The results of the present study demonstrated that capsaicin-meditated glycolysis inhibition in ESCC cells was associated with its effect on phosphatase and tensin homolog (PTEN) and subsequent PTEN-mediated HK-2 inhibition.

Materials and methods

Cell line and reagents. Het-1A cell was purchased from American Type Culture Collection (ATCC), 293T, KYSE150, KYSE410 and KYSE510 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Het-1A and HEK 293T cells were cultured with DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and KYSE150, KYSE410 and KYSE510 cells were cultured with RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin in a 37˚C incubator with 5% CO_2. Capsaicin (cat. no. 03813) and anti-β-actin (cat. no. A5316) antibody were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Anti-rabbit immunoglobulin (Ig)G-horseradish peroxidase (HRP) (cat. no. sc-2004) and anti-mouse IgG-HRP (cat. no. sc-2005) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-HK-2 (cat. no. 2867), anti-PTEN (cat. no. 9188), anti-phosphorylated (p)-RAC-α serine threonine-protein kinase (Akt) (Ser473; cat. no. 4060) and anti-p-Akt (Thr308; cat. no. 13038) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Lentiviral plasmid pLKO.1-short hairpin green fluorescent protein (shGFP) (cat. no. 30323) was obtained from Addgene, Inc. (Cambridge, MA, USA); pLKO.1-shPTEN#1, (cat. no. TRCN0000028991) and pLKO.1-shPTEN#2 (cat. no. TRCN0000028989) were obtained from Thermo Fisher Scientific, Inc.

Cell proliferation assay. Cells were seeded (2,000 cells/well) in 96-well plates and cultured for 24 h. Following treatment with different concentrations of capsaicin (30, 60 and 120 μM), the plates were cultured in a 5% CO_2 incubator at 37˚C. At various time points (0, 24, 48 or 72 h), 20 μl/well CellTiter96 Aqueous One Solution (Promega Corporation, Madison, WI, USA) was added and incubated at 37˚C for 1 h, and the absorbance was measured at 490 nm by SpectraMax microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Western blotting. Cells were harvested by trypsinization and pelleted by centrifugation at 300 x g for 5 min at room temperature. The pellets were lysed in NP40 lysis buffer [50 mmol/l Tris-HCl (pH 8.0); 150 mmol/l NaCl; 0.5% NP40] supplemented with protease cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein samples (10 μg/lane) were subjected to 10% SDS-PAGE and subsequently electrically transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Following blocking in 5% non-fat dry milk in TBS at room temperature for 1 h., the membranes were probed with specific primary antibodies (1:100 dilution) overnight at 4˚C, washed three times with TBS-Tween 20, and incubated with HRP-conjugated secondary antibodies (1:2,000 dilution) at room temperature for 1 h. The membranes were washed with TBS-Tween-20 and the protein bands were visualized using enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Measurement of glucose uptake and lactate production. Tumor cells were exposed to varying concentrations of capsaicin for 24 h, and subsequently trypsinized and seeded in 6-well plates ($5x10^5$ cells/well). Following incubation for 4 h at 37˚C, media were discarded and cells were incubated in fresh culture medium for a further 8 h at 37˚C. Glucose and lactate levels were measured using the Automatic Biochemical Analyzer (AU680, Beckman Coulter International, Brea, CA, USA). The relative glucose consumption rate and lactate production rate were normalized by the protein concentration of the samples.

Lentiviral infection. KYSE150 cells ($2x10^6$) were seeded in 10-cm dishes and pLKO.1-shp53 was co-transfected into 293T ($5x10^6$) cells together with PSPAX2 and PMD2-G at 37˚C. A total of 48 h subsequent to transfection, viral supernatant fractions were collected and infected into KYSE150 cells with 10 μg/ml polybrene. 24 h subsequent to infection, the medium was replaced with fresh medium containing 0.5 μg/ml puromycin (cat. no. S7417; Selleck Chemicals, Shanghai, China). Further experiments were performed with these cells until the control cells (without infection) completely died (2-3 days) in the puromycin medium.

Statistical analysis. All statistical analysis was performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). The experiments were performed in triplicate. All the quantitative data are expressed as the mean ± standard deviation. The significant differences between two groups were assessed using a two-tailed Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Capsaicin inhibits ESCC cell proliferation in vitro. The efficacy of capsaicin against ESCC cell proliferation was investigated *in vitro*. In three ESCC cell lines, KYSE150, KYSE510 and KYSE410, capsaicin demonstrated an inhibitory effect on cell growth. At a low concentration (30 μM), exposure to capsaicin led to little growth inhibition in these three cell lines. With an increase of capsaicin concentration and the duration of treatment with capsaicin, cell growth was markedly repressed. As presented in Fig. 1, in all tested cell lines, at a high concentration (120 μM) for 72 h, cell proliferation was inhibited by >50% compared with the control group. The results of the present study demonstrated that capsaicin exhibited antitumor activity in ESCC cells *in vitro.*

HK-2 decreases following treatment with capsaicin in ESCC cells. Aerobic glycolysis is one of the metabolic characteristics of tumor cells, and is important for the survival and growth of cancer cells. In tumor cells, particularly those with a highly glycolytic phenotype, HK-2 has been reported to be overexpressed; however, its expression in ESCC cells was unknown. Therefore, the expression of HK-2 was investigated

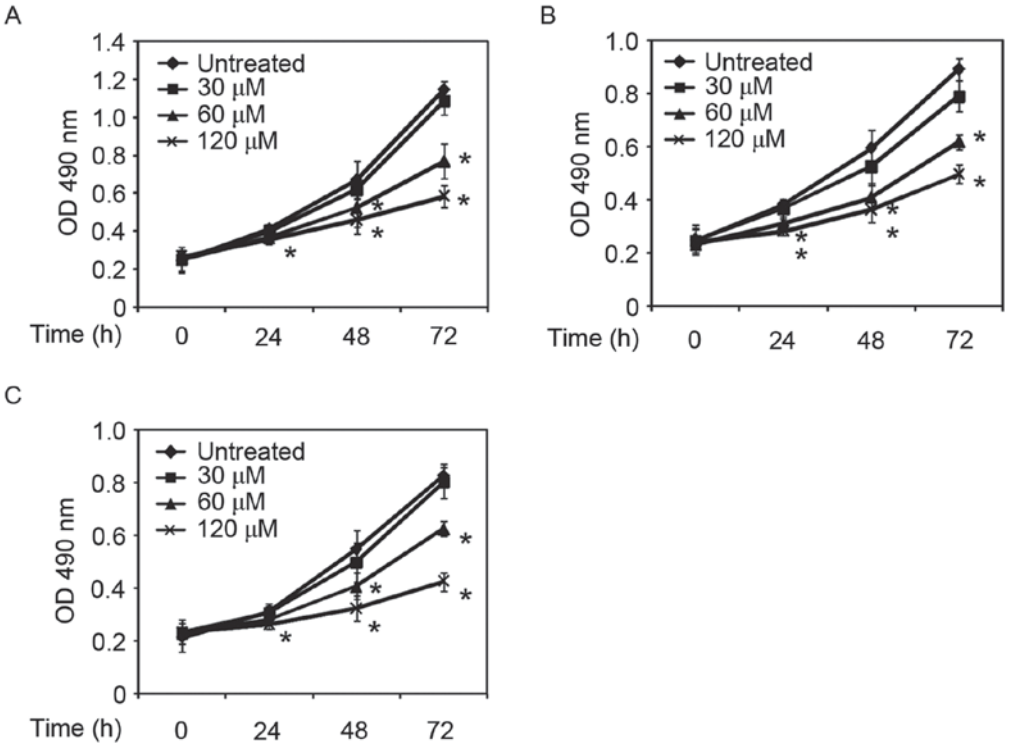

Figure 1. Capsaicin inhibits the proliferation of ESCC cancer cells *in vitro*. Human ESCC cancer cell lines (A) KYSE150, (B) KYSE510 and (C) KYSE410, were treated with the indicated concentrations of capsaicin for 0, 24, 48 or 72 h. Cell proliferation was analyzed by absorbance at OD_{490}. A decrease of ESCC cell proliferation was observed following treatment with capsaicin. *P<0.05 vs. the untreated cells at the respective time points. ESCC, esophageal squamous cell carcinoma; OD, optical density.

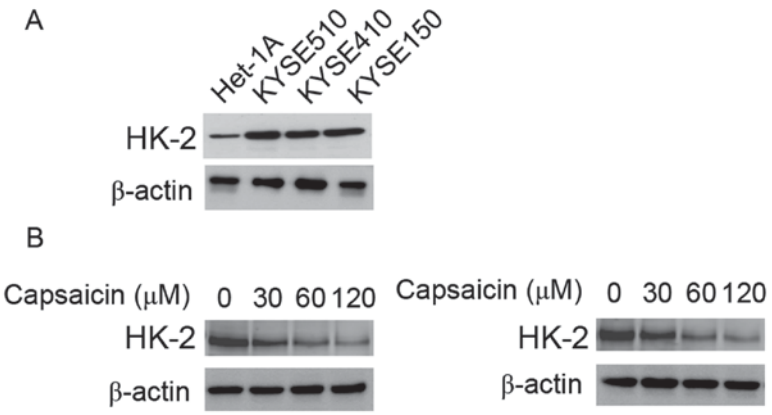

Figure 2. Capsaicin suppresses HK-2 expression in ESCC cancer cells. (A) HK-2 expression in different ESCC cells. The cell lysates of ESCC cells were subjected to SDS-PAGE and probed with HK-2 antibody. (B) KYSE150 (left) and KYSE510 (right) cells were treated with various concentrations of capsaicin and the cell lysates were subjected to SDS-PAGE to examine the alteration in HK-2 expression. β-actin was used as loading control. HK-2, hexokinase-2; ESCC, esophageal squamous cell carcinoma.

in three ESCC cell lines. As presented in Fig. 2A, compared with the normal esophageal epithelial cell line Het-1A, the expression level of HK-2 in the three ESCC cells was increased, suggesting that tumor glycolysis in these ESCC cells is highly active. The effect of capsaicin on HK2 expression was subsequently investigated. As presented in Fig. 2B, in KYSE150 and KYSE150 cells, the expression of HK-2 was suppressed by capsaicin in a concentration dependent manner. At a concentration of 120 μM, the expression of HK-2 was markedly decreased compared with the control group.

Capsaicin suppresses glucose uptake and lactate secretion in ESCC cells. It is known that HK-2 serves a role in the process of tumor glycolysis. Due to the effect of capsaicin on HK-2 expression, it was hypothesized that capsaicin may exhibit an inhibitory effect on tumor glycolysis. Glucose uptake and lactate secretion are indicators of tumor glycolysis; therefore, the effect of capsaicin on glucose uptake and lactate secretion was investigated. As presented in Fig. 3A, KYSE150 cells treated with capsaicin (60 μM) demonstrated decreased glucose uptake compared with control cells. In KYSE510 cells, capsaicin repressed glucose consumption in a dose-dependent

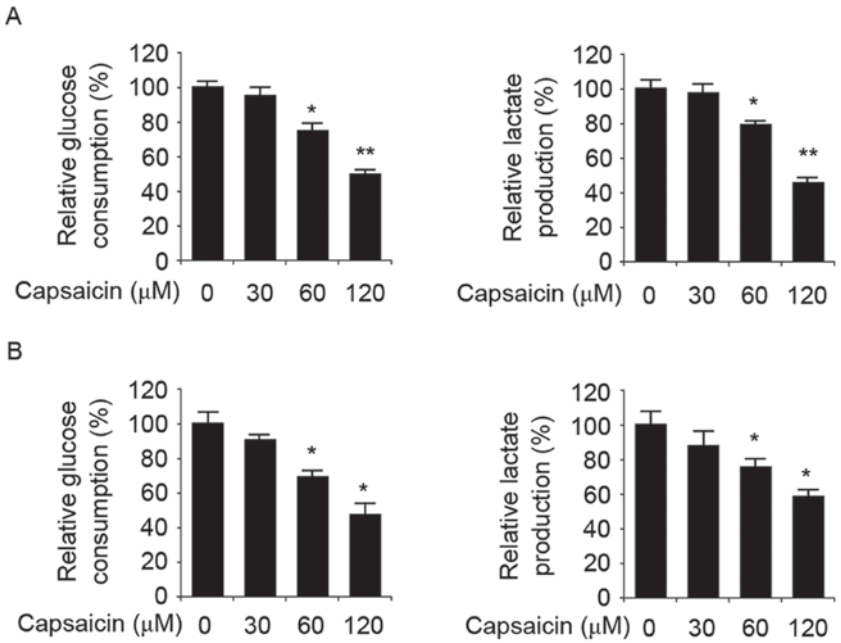

Figure 3. Capsaicin suppresses tumor glycolysis in esophageal squamous cells carcinoma cells. (A) KYSE150 and (B) KYSE510 cells were treated with various concentrations of capsaicin. Glucose consumption (left panels) and lactate production (right panels) were analyzed using the Automatic Biochemical Analyzer. The graph presents the data of ≥3 independent experiments expressed as the mean ± standard deviation. Data were analyzed using a Student's t-test. *P<0.05, **P<0.01 vs. 0-μg treatment group.

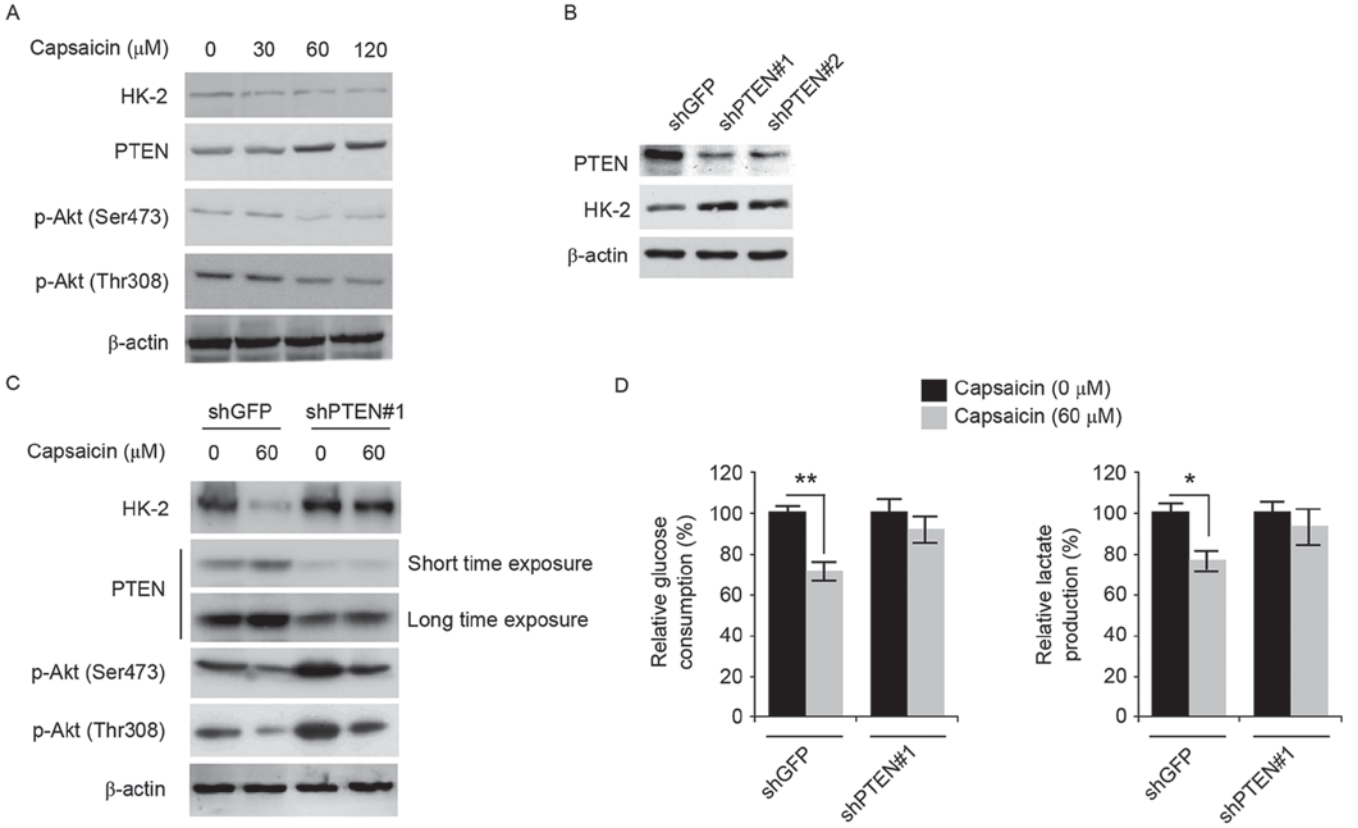

Figure 4. Capsaicin-mediated glycolysis inhibition is associated with PTEN. (A) The effect of capsaicin on the expression of PTEN and Akt phosphorylation. KYSE150 cells were treated with various concentrations of capsaicin and the alteration of the indicated protein was probed with corresponding antibodies. (B) Knockdown of PTEN expression in KYSE150 cells with specific PTEN shRNA. PTEN knockdown was validated using western blotting. (C) The effect of capsaicin on HK-2 expression in PTEN-knockdown KYSE150 cells. KYSE150 cells with GFP shRNA or PTEN shRNA were treated with 60 μM capsaicin for 24 h and cell lysates were subjected to western blotting with corresponding antibodies. (D) The effect of capsaicin on tumor glycolysis in PTEN-knockdown KYSE150 cells. KYSE150 cells were transfected with GFP shRNA or PTEN shRNA and treated with 60 μM capsaicin for 24 h. Glucose consumption (left panels) and lactate production (right panels) was analyzed. The graph presents the data of ≥3 independent experiments expressed as the mean ± standard deviation. The data were analyzed using a Student's t-test. *P<0.05, **P<0.01. PTEN, phosphatase and tensin homolog; HK-2, hexokinase-2; shRNA, small hairpin RNA; GFP, green fluorescent protein; Akt, RAC-α serine threonine-protein kinase; p, phosphorylated.

manner. In addition to the suppression of glucose consumption, the secretion of lactate, which is the product of tumor glycolysis, was decreased. In KYSE150 and KYSE510 cells, treatment with 60 μM capsaicin resulted in a notable reduction of lactate production in the supernatant compared with the control group.

Capsaicin mediates glycolysis inhibition in a PTEN dependent manner. As presented in Fig. 4A, in KYSE150 cells, the expression of PTEN, which negatively regulates the phosphatidylinositol 3-kinase/Akt signaling pathway in tumor cells, was increased in a dose-dependent manner following treatment with capsaicin. In addition to an increase in PTEN expression, phosphorylation of Akt at Ser473 and Thr308 was suppressed. In order to investigate the role of PTEN in facilitating capsaicin-mediated inhibition of glycolysis, PTEN shRNA was developed to knock down the expression of PTEN in KYSE150 cells. Following transfection of KYSE-150 cells with PTEN shRNA, the expression of PTEN was decreased compared with the control group (shGFP group), which validated the efficiency of the shRNA used (Fig. 4B). In addition to the knockdown of PTEN, the expression of HK-2 was increased. The efficacy of capsaicin was assessed in PTEN-knockdown KYSE-150 cells, as presented in Fig. 4C; in PTEN-knockdown cells, the expression of HK-2 was unaltered following treatment with capsaicin. In addition, the phosphorylation of AKT at Ser473 and Thr308 was increased compared with the control group. The effect of PTEN knockdown on glucose uptake and lactate secretion was additionally investigated. As presented in Fig. 4D, in PTEN-knockdown KYSE150 cells, glycolysis inhibition caused by 60 μM capsaicin was attenuated. Glucose uptake and lactate production in PTEN shRNA cells was markedly recovered compared with the shGFP group, which suggested an important role for PTEN in capsaicin-mediated glycolysis inhibition.

Discussion

Esophageal cancer is the eighth most common cancer and the sixth leading cause of mortality from cancer worldwide. In Asia, particularly in China, ESCC is the predominant type. Despite advances in cancer surgery and chemotherapy, the five-year survival rate of patients with late-stage esophagus carcinoma is relatively low (21). Therefore, there is a requirement to discover and develop chemical entities with novel antitumor mechanisms against esophagus carcinoma. Capsaicin, a component of red peppers which is widely consumed, has been reported to exert chemopreventive and chemotherapeutic activities in various types of cancer; however, its effect on tumor glycolysis remains unknown. The results of the present study demonstrated that capsaicin exhibited an inhibitory effect on tumor glycolysis in ESCC by downregulating HK-2 expression. Further investigations revealed that PTEN was involved in the capsaicin-mediated inhibition of tumor glycolysis in ESCC cells.

Clinical studies have demonstrated that the level of glycolysis in tumor tissue, which can be detected with ^{18}F-FDG-PET/computerized tomography technology, is a useful prognostic factor for patients with ESCC (22,23). The conversion of glucose to glucose-6-phosphate, an irreversible step in glycolysis, is mediated by HK-2. HK-2 localizes to the outer mitochondrial membrane protein voltage-dependent anion channel, where it gains preferential access to the ATP generated by the mitochondria and protection from inhibition by glucose-6-phosphate (24). Therefore, HK-2 is reported to be overexpressed in various types of cancer, particularly those with a highly glycolytic phenotype. In the present study, the overexpression of HK-2 was identified in ESCC cells. Compared with normal esophageal epithelial cells, HK-2 expression in ESCC cells was increased, suggesting that the glycolysis level in the ESCC cells was increased. In capsaicin-treated ESCC cells, the expression of HK-2 was decreased. In addition, the uptake of glucose and the secretion of lactate by ESCC cells was reduced. The results of the present study demonstrated that capsaicin effectively inhibited tumor glycolysis. A previous study indicated that HK-2 was necessary for the tumorigenicity of non-small cell lung cancer and breast cancer in humans, whereas HK2 deletion resulted in rapid suppression of tumor growth (25). The proliferation of ESCC cells was suppressed by capsaicin *in vitro* in the present study. Certain studies have demonstrated that the activity of HK-2 was associated with chemoresistance (26). The sensitivity of tumor cells to chemotherapy has been demonstrated to be enhanced through the inhibition of glycolysis by targeting HK-2 (27,28). Due to the effect of capsaicin on HK-2 and tumor glycolysis, capsaicin may be able to increase the efficacy of other chemotherapies.

Following treatment with capsaicin, the expression of PTEN, which has been identified to be a tumor suppressor, was increased in a dose-dependent manner. In addition, the phosphorylation of Akt at Ser473 and Thr308 was decreased. As reported by Wang *et al* (29), HK-2 was selectively upregulated by the combined loss of PTEN and p53 in prostate cancer cells; the PTEN deletion increased HK-2 mRNA translation through the activation of the Akt-methylated target of rapamycin complex 1-4-Erb-binding protein 1 axis. In order to investigate the role of PTEN in capsaicin-mediated glycolysis inhibition, PTEN expression in KYSE150 cells was knocked down using shRNA in the present study. In PTEN-knockdown cells, glycolysis inhibition mediated by capsaicin was attenuated, suggesting that PTEN facilitates the effect of capsaicin on tumor metabolism. As a tumor suppresser, loss or mutations of PTEN have been identified in various types of cancer. Clinical evidence has demonstrated that PTEN expression in ESCC was an important prognostic indicator; the 5-year survival rate in patients with PTEN-positive expression was 82%, compared with 39% in patients with PTEN-negative expression (30). Consistent with previous reports, in the present study, the expression of HK-2 was increased in PTEN-knockdown cells compared with control cells. Therefore, in patients with ESCC, aberrant tumor glycolysis may be a reason for the poor prognosis associated with PTEN loss.

In conclusion, the present study demonstrated that capsaicin exhibited an inhibitory effect on tumor glycolysis by decreasing HK-2 expression in ESCC cells. Further investigation demonstrated that the inhibition of glycolysis mediated by capsaicin was associated with an increase of PTEN expression following treatment with capsaicin. The present study provides a novel mechanism to elucidate the antitumor activity of capsaicin.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XLM, HYZ, DHL and YZ designed the experiments. XLM and HYZ carried out the majority of the experimental work. LPY and HFY performed the glucose and lactate assays. JSZ and YZ were responsible for shRNA construction. XLM, HYZ, DHL and YZ analyzed the data. XLM, HYZ and YZ wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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