Butein Inhibited In Vitro Hexokinase-2-Mediated Tumor Glycolysis in Hepatocellular Carcinoma by Blocking Epidermal Growth Factor Receptor (EGFR)

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Background: Anaerobic glycolysis is an important physiological process of all cancer cells. Butein has been reported to demonstrate substantial antitumor activities in various cancers. However, the effect of butein on tumor glycolysis remains unclear. In this study, the effect of butein on tumor glycolysis and the underlying mechanism were investigated in hepatocellular carcinoma (HCC).

Material/Methods: Cell proliferation assay and anchorage-independent growth assay were carried out to evaluate the antitumor activities of butein in vitro. The effect of butein on tumor glycolysis was determined by examining the changes in glucose uptake and lactate production. Hexokinase-2 (HK-2) expression in HCC cells upon butein treatment was analyzed by Western blotting. The activity of butein on EGFR signaling pathway was studied and its potency in EGFR exogenous overexpression cells was investigated.

Results: After butein treatment, HCC cell proliferation was significantly inhibited (91.4% in Hep3B and 88.2% in Huh-7 at 80 μM, p<0.001). Moreover, the number of colonies formed in the agar was substantially decreased (93.8% in Hep3B and 72.3% in Huh-7 at 80 μM, p<0.001). With the suppression of HK-2 expression, glucose consumption in Hep3B and Huh-7 cells decreased by 48.4% and 56.3%, respectively (p<0.01), and the lactate production also was reduced accordingly (39.5% in Hep3B and 48.6% in Huh-7, p<0.01). Mechanism investigations demonstrated that butein dose-dependently blocked the activation of the EGFR signaling pathway in HCC cells. In EGFR exogenous overexpression cells, the glycolysis suppression exerted by butein was substantially attenuated.

Conclusions: Butein has a substantial inhibitory effect on tumor glycolysis in HCC cells, and the glycolysis suppression exerted by butein is closely related to its effect on the EGFR signaling pathway.

MeSH Keywords: Carcinoma, Hepatocellular • Genes, erbB-1 • Glycolysis • Hexokinase

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Background

As opposed to normal cells in which glucose is completely oxidized to CO₂ and H₂O via the Krebs cycle in the presence of oxygen, the glucose in tumor cells is converted into pyruvate and lactate, even when sufficient oxygen is available, which is termed the Warburg effect or aerobic glycolysis [1,2]. The glycolysis in tumor cells not only provides energy to support the rapid proliferation, it also satisfies the huge metabolic needs in macromolecule synthesis [3]. In the process of glucose metabolism, glucose is firstly phosphorylated by hexokinases (HKs) and is turned into glucose-6-phosphate (G-6P), which is an essential irreversible rate-limiting step [4]. So far, 4 different isoforms of hexokinases – HK1–4 – have been characterized [5]. HK-2 is considered to be the principal regulated isoform in different cell types [6]. The analysis of clinical specimens demonstrated that HK2 overexpression occurs in a variety of cancers, including breast cancer [7], gastric cancer [8], esophageal carcinoma [9], and cervical cancer [10]. In HCC, HK-2 was found to be overexpressed in 55.67% of clinical specimens and was closely correlated with the poor survival of patients [11]. It was also demonstrated that the hyperactive glycolysis in HCC cells was closely related to high HK-2 expression [12]. Thus, it is an effective method for the clinical treatment of HCC patients by interfering with tumor glycolysis [13,14]. 3-bromopyruvate (3-BP), which suppresses the tumor glycolysis via directly blocking HK-2 activity, has been designated by the FDA as an orphan drug and was reported to show promising efficacy and to substantially prolong the lives of HCC patients [15].

Butein is a polyphenolic compound isolated from a number of plants, such as Caragana jubata and Rhus verniciflua [16]. Like any other compounds in the flavonoid family, butein displays the multiple biological activities of this family. Butein was reported to provide health benefits, such as disease prevention, antioxidant activities, and chemoprevention [17,18]. Moreover, butein has been found to demonstrate anti-inflammatory, anti-restenosis, and anti-adipogenic activities [19–21]. Recently, butein has been reported to have profound antitumor activities in different cancer models, such as acute lymphoblastic leukemia [14], ovarian cancer [22], cervical cancer [23], and breast cancer [24]. A variety of proteins, including STAT3, ERK, JNK, Akt, and p38, were reported to be altered after butein treatment [25–27]. Through NF-κB inactivation, butein was demonstrated to prevent tumor cell invasion and migration [28]. In addition, it was also reported that butein blocked cancer metastasis via repressing urokinase plasminogen activator (uPA) [29].

In the present study, the activities of butein against tumor glycolysis and its underlying mechanisms were investigated. After butein treatment, HK-2 expression was substantially decreased and the tumor glycolysis in HCC cells was significantly suppressed. Moreover, we demonstrated that the glycolysis inhibition caused by butein was closely related to inhibition of the EGFR signaling pathway. Our study shows a novel mechanism by which butein exerts its antitumor activity in HCC, and provides a preclinical basis for translational clinical studies to determine if butein has the potential for HCC treatment.

Material and Methods

Cell culture and reagents

Hep3B and the human embryonic kidney cell 293T were obtained from American Type Culture Collection (Manassas, VA, USA). Huh-7 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Hep3B, 293T, and Huh7 cells were cultured with Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. Butein, β-actin (AS5161) antibody, and the chemical reagents used in this study were products of Sigma-Aldrich (St. Louis, MO, USA). The antibodies of HK2 (#2867), VDAC1(#4866), BCL2 (#2870), BCL-XL (#2764), MCL-1 (#5453), cleaved PARP (#5625), cleaved caspase-3 (#9664), EGFR (#4267), p-EGFR-Tyr1068 (#3777), p-Akt-Ser473 (#4060), p-ERK1/2-Thr202/Tyr204 (#8544), anti-rabbit IgG HRP (#5704), and anti-mouse IgG HRP (#7076) were obtained from Cell Signaling Technology (Beverly, MA, USA). Lentivirus plasmids (pLKO.1-shEGFR) were products of Thermo Scientific (Huntsville, AL, USA). Lipofectamine™ 2000 was obtained from Invitrogen (Carlsbad CA, USA).

Cell proliferation assay

HCC cells were digested with trypsin, and 100 μl HCC cell suspensions (2×10⁴ cells) were added into 96-well plates. After 24 h, HCC cells were treated with different concentrations of butein. At different time points (24, 48, and 72 h), cell viability was examined by MTS assay (Promega, Madison, WI, USA).

Anchorage-independent cell growth assay

We melted 1% agar and cooled it to 40°C, then mixed it with equal volumes of 2X DMEM culture medium containing 20% FBS. The 1.5-ml mixture was added into 6-well plates and set aside for 5 min to allow the agar to solidify, then 100 μl cell suspension (8×10⁴ cell) with 1.5 ml 0.3% Basal Medium Eagle agar supplemented with 10% FBS and different concentrations of butein was added. The plates were incubated at 37°C in a 5% CO₂ incubator for 2 weeks and the cell culture medium was replaced 1–2 times per week. The colonies formed in the agar were counted by using a microscope.
Tumor glycolysis measurement

The glucose consumption and lactate production in cell culture medium were examined. The HCC cells were digested with trypsinized and 5×10^4 cells/well were plated into 6-well plates. After the cells were attached to the plate (12 h), the cells were washed with PBS and cultured with 1 ml fresh medium containing different concentrations of butein for 8 h, then the cell culture medium was collected and the amounts of glucose and lactate were assessed using the Automatic Biochemical Analyzer (7170A, HITACHI, Tokyo, Japan). The HCC cells were lysed with RIPA buffer and the protein concentrations were determined. The relative rates of glucose consumption and lactate secretion were normalized by the protein concentration.

Mitochondrial isolation

After butein treatment, HCC cells were harvested and the mitochondria fractions were isolated using the Mitochondria Isolation Kit (Biovision, San Francisco, CA, USA) following the manufacturer’s directions. Briefly, HCC cell pellets were washed with cold PBS and suspended in the isolation buffer. After incubation on ice for 2 min, HCC cells were stroked with a glass homogenizer 3–4 times and the homogenate was centrifuged once at 600 g for 10 min at 4°C. The supernatants were harvested and then pelleted by centrifugation at 7000 g for 10 min. The pellets were washed with isolation buffer and then lysed with the appropriate amount of RIPA buffer.

Western blotting

HCC cells treated with butein were lysed with RIPA buffer on ice for 30 min, then the lysates were collected and centrifuged at 12,000 g for 10 min. The supernatants were harvested and the protein concentrations were determined with the BCA assay reagent (Pierce, Rockford, IL, USA) following manufacturer’s directions. The protein samples (10 μg/lane) were separated with 10% SDS-PAGE and then transferred to a PVDF membrane. The nonspecific binding sites on the membranes were blocked with 5% non-fat milk at room temperature for 1 h, then the membranes were incubated with indicated primary antibodies at 4°C overnight. After washing with PBST, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. After washing, the bands on the membrane were visualized using an enhanced chemiluminescence detection kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer’s instructions.

Lentiviral infection

The 293T cells were co-transfected with pLKO.1-sh-GFP or pLKO.1-sh-EGFR lentivirus plasmid, together with PSPAX2 and PMD2-G using lipofectamine 2000. After 48 h, the supernatants were collected and filtered through a 0.45-μm filter. The viral supernatants were infected into Hep3B cells with 10 μg/mL polybrene. After 24 h, the medium was discarded and replaced with fresh medium containing 1 μg/ml puromycin. Once the control cells (without infection) were completely died (about 6 days) in the puromycin medium, further experiments were performed with these infected cells.

Statistical analysis

The data was presented as mean ±SD and the statistical analyses were performed by using Statistics Package for Social Science (SPSS) software (version 16.0). P values <0.05 were considered to be statistically significant (t test).

Results

Butein suppressed HCC cell proliferation in vitro

Firstly, the activity of butein against cell proliferation was examined in Hep3B and Huh-7 cells using MTS assay. As shown in Figure 1A, a significant dose- and time-dependent proliferation inhibition were observed; the proliferation inhibition rate reached 91.4% in Hep3B and 88.2% in Huh-7 at 72 h after treatment with 80 μM butein (n=3, p<0.001). In addition to the cell proliferation inhibition, the anchorage-independent growth in soft agar was also inhibited. After butein treatment, the number of colonies formed was significantly decreased (93.8% in Hep3B and 72.3% in Huh-7 at 80 μM, n=3, p<0.001), and fewer colonies were observed (Figure 1B).

Butein inhibited tumor glycolysis via reducing HK-2 expression

Next, we assessed the effect of butein on tumor glycolysis by examining the status of glucose uptake and lactate secretion. As shown in Figure 2A, 2B, the glucose consumption was significantly decreased in a dose-dependent fashion (48.4% and 56.3% in Hep3B and Huh-3 cells, respectively, at 80 μM, n=4, p<0.01). With the reduction of glucose absorption, the amount of lactate produced by HCC cells was dramatically decreased (48.4% and 46.8% in Hep3B and Huh-7 at 80 μM, n=4, p<0.01). After incubation with butein, the expression of HK-2 in Hep3B and Huh-7 cells was dose-dependently reduced in the whole-cell lysate (Figure 2C). Given that HK-2 is mainly located in the outer membrane of mitochondria to mediate tumor glycolysis, we examined the expression of HK-2 in mitochondria. Consistent with the results in whole-cell lysate, HK-2 in mitochondria was also substantially decreased (Figure 2D).
Butein induced apoptosis in HCC cells

In addition to its effect on tumor glycolysis, butein also induced apoptosis in HCC cells. The expression of cleaved caspase-3 and PARP were significantly increased in a dose-dependent manner by butein (Figure 3A). The expression of proteins which antagonize cell apoptosis and promote cell survival were significantly decreased after butein treatment (in Hep3B cells, the expression of Bcl-2, Bcl-xl, and Mcl-1 decreased by 91.3%, 42.3%, and 70.5%, respectively, and expression in Huh-7 cells decreased by 80.4%, 90.3%, and 24.8%, respectively, after treatment with 80 μM butein, n=3, p<0.01, Figure 3B).

Butein inhibited EGFR signaling pathways in HCC cells

In Hep3B cells, the activity of EGFR was blocked by butein in a dose-dependent manner, and at 80 μM EGFR phosphorylation was substantially inhibited (80%, n=3, p<0.001). With the inactivation of EGFR, the activity of downstream signaling pathway ERK1/2 and Akt were both inhibited (67% inhibition for ERK1/2 and 71.4% for Akt, n=3, p<0.001, Figure 4A). To confirm the inhibition of EGFR by butein, we used EGF to induce EGFR activation and then tested the effect of butein. As shown in Figure 4B, after EGF stimulation, the phosphorylation of EGFR was substantially increased. After the incubation with 20 μM butein for 60 min, EGF-induced activation of
Figure 2. Butein suppressed tumor glycolysis in HCC cells via reducing HK-2 expression. (A, B) Hep3B (A) and Huh-7 (B) cells were treated with butein and the amounts of glucose and lactate in cell culture medium were examined as described (n=4). (C, D) Hep3B (left) and Huh-7 (right) cells were incubated with butein and HK-2 expression in the whole-cell lysates (C) or the mitochondrial fractions and (D) was examined by Western blotting (n=4). The asterisks (* p<0.05, ** p<0.01, *** p<0.001, Student's t test) indicate a significant difference.
Figure 3. Butein induced HCC cell apoptosis and decreased the expression of proteins in BCL-2 family. (A, B) Hep3B (left) and Huh-7 (right) cells were treated with different concentrations of butein for 24 h, the cell lysates were collected and probed with indicated antibodies (n=3). The asterisks (* p<0.05, ** p<0.01, *** p<0.001, Student’s t test) indicate a significant difference.
**Figure 4.** Butein inhibited EGFR signaling pathway in HCC cells. (A) Hep3B cells were exposed to different concentrations of butein for 2 h, the cell lysates were subjected to Western blotting to examine indicated proteins (n=3). (B) Hep3B cells were starved overnight and incubated with 20 μM butein for 2 h. After stimulation with 100 ng/ml EGF for indicated times, the cell lysates were probed with indicated antibodies (n=3). The asterisks (* p<0.05, ** p<0.01, *** p<0.001, Student’s t test) indicate a significant difference.
**Figure 5.** Overexpression of EGFR attenuated the glycolysis suppression mediated by butein. (A) Hep3B cells were transfected with EGFR shRNA and EGFR expression was examined by Western blotting (n=3). (B) HK-2 expression in EGFR knockdown Hep3B cells was examined (n=3). (C, D) Exogenous overexpression of EGFR attenuated butein-mediated glycolysis suppression. Hep3B cells were transfected with pcDNA3.0-Flag-EGFR or blank vector (vehicle) for 24 h and treated with 40 μM butein for 24 h, the expression of indicated protein were analyzed by Western blotting (C) and the glucose uptake and lactate secretion were examined (D) (n=4). The asterisks (* p<0.05, ** p<0.01, *** p<0.001, Student’s t test) indicate a significant difference.
EGFR was inhibited by 49.7% (n=3, p<0.01), the phosphorylation of ERK1/2 and Akt were also suppressed in a time-dependent manner, and the activity of ERK1/2 and Akt decreased by 41.2% and 48.3%, respectively, at 60 min (n=3, p<0.001).

Overexpression of EGFR impaired butein-mediated glycolysis suppression

To investigate the relationship between EGFR and HK-2 and whether the decrease in HK-2 expression was due to blockade of the EGFR signaling pathway, we first developed EGFR shRNA to knock down the expression of EGFR in HCC cells (Figure 5A). After EGFR shRNA transfection, EGFR expression was significantly decreased compared to the control group (70.3%, 65.4%, 38.6%, and 1%, respectively, for the 4 different EGFR shRNAs, n=3). Consistent with the reduction in EGFR, HK-2 expression was also substantially decreased (68.1% for #1 shRNA and 63.5% for #2 shRNA, n=3, p<0.001), suggesting that EGFR is involved in the regulation of HK-2 (Figure 5B). To further elucidate the role of EGFR in butein-mediated glycolysis suppression, EGFR was overexpressed in Hep3B cells, and then we examined the effect of butein on tumor glycolysis. As shown in Figure 5C, in contrast to the control group, after the transfection of pcDNA3.0-Flag EGFR, the activity of EGFR was increased by about 75% (n=4, p<0.001). With EGFR activation, HK-2 expression was substantially increased (65.1%, n=4, p<0.001), and the suppression of glucose uptake and lactate production were significantly recovered (22% for glucose consumption and 16% for lactate production, n=4, p<0.001, Figure 5D).

Discussion

Hepatocellular carcinoma (HCC) is the seventh most common solid tumor and the second leading cause of cancer-related death in the world [30]. In contrast to the high mortality, the effective systemic therapies for HCC treatment, especially the targeted therapy with high efficiency and low toxicity, are still limited clinically. Therefore, it is urgent to discover and develop novel candidate agents for HCC therapy. In this study, we demonstrated that butein exerted profound antitumor effects against HCC via suppressing tumor glycolysis. Mechanism investigations revealed EGFR-mediated HK-2 reduction was an important underlying mechanism by which butein exerts its effect in tumor glycolysis.

The glycolysis in malignant tumor cells not only provides the main energy source to support cell proliferation, but also provides the products generated in this process, such as lactate, contributing to a microenvironment which favors tumor development, invasion, metastasis, escape from immune surveillance, chemotherapy resistance, and tumor recurrence [31,32]. Therefore, the 18F-FDG PET/CT technology, which can examine the status of glucose metabolism in tumor cells, is widely used for cancer diagnosis in clinical applications. Na et al. reported that 18F-FDG uptake had an incremental prognostic value for overall survival in advanced HCC patients [33]. After butein treatment, owing to the decrease in HK-2, the glucose consumption and lactate production in HCC cells were significantly decreased (Figure 2), resulting in the inhibition of HCC cell proliferation and anchorage-independent growth (Figure 1). In addition to taking part in the regulation of tumor glycolysis, HK-2 also interacts with the voltage-dependent anion channel (VDAC) on the outer membrane of mitochondria to prevent cell apoptosis [34]. With the decrease in HK-2 in mitochondria, outer mitochondria membrane integrity was disrupted and apoptosis of HCC cells was induced by butein, which was confirmed by the increase of cleaved PARP and caspase-3 (Figure 3). In addition to the reduction of HK-2, proteins in the BCL-2 family, such as Bcl-xl, Bcl-2 and Mcl-1, which protect tumor cells against pro-apoptotic signals and maintain the integrity of the outer mitochondrial membrane [35], were also prominently downregulated by butein and facilitated HCC cell apoptosis.

The EGFR signaling pathway in cancer cells has been intensively studied and has been shown to play a key role in controlling tumor cell proliferation, differentiation, and survival. Overexpression of EGFR was found in about 68% of human HCC and is closely related to tumor metastasis and poor survival of patients [36]. Consistent with the results reported by Jung et al. [37], butein demonstrated a significant inhibitory effect on EGFR activity in HCC cells. EGFR and its downstream signaling pathways were inactivated in a time and dose-dependent manner (Figure 4). The decrease in HK-2, and the glycolysis suppression caused by butein, were significantly impaired by exogenous EGFR overexpression (Figure 5C-D), suggesting that blockade of the EGFR signaling pathway is an important underlying mechanism by which butein exerts potent effects against tumor glycolysis. The proteome mapping of epidermal growth factor (EGF)-induced hepatocellular carcinomas identified that there was a significant change in energy production, which relied on the glycolysis rather than the mitochondrial oxidative phosphorylation [38]. As the first rate-limiting enzyme of glycolysis, HK-2 expression is regulated by EGFR.

Conclusions

This study found that through the inhibition of HK-2-mediated glycolysis, butein demonstrated profound antitumor activities against HCC in vitro. Further investigations suggested that inhibition of the EGFR-HK-2 signaling pathway was an important mechanism of the glycolysis suppression mediated by butein. Our experiments provide the preclinical rationale for the clinical application of butein in HCC patients.
Conflict of interest

The authors have declared that no competing interest exists.

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