PI3K/Akt is involved in bufalin-induced apoptosis in gastric cancer cells

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Bufalin is the active ingredient of the Chinese medicine Chan Su, and it has been reported that bufalin induces apoptosis in some human leukemia and solid cancer cell lines. The exact mechanism of bufalin-induced apoptosis is, however, still not clear. In this study, we demonstrated that bufalin inhibited the proliferation of gastric cancer MGC803 cells in a dose-dependent and time-dependent manner. At a low concentration (20 nmol/l), bufalin induced M-phase cell cycle arrest, whereas at a high concentration (80 nmol/l) it induced apoptosis in MGC803 cells. Bufalin increased the Bax/Bcl-2 ratio and activated caspase-3 during the apoptotic process of MGC803 cells. It should be noted that bufalin transiently activated the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and then inhibited it completely, and upregulated the Casitas B-lineage lymphoma (Cbl) family of ubiquitin ligases, upstream modulators of PI3K. A combination of bufalin and LY294002, a PI3K-specific inhibitor, enhanced apoptosis, but PD98059, an extracellular-regulated protein kinase-specific inhibitor, had no significant effect on bufalin-induced apoptosis. These results suggested that the PI3K/Akt pathway might play a key role in bufalin-induced apoptosis in gastric cancer MGC803 cells. *Anti-Cancer Drugs* 20:59–64 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Gastric cancer is the second most common cancer in the world [1], and still remains one of the major causes of cancer deaths in China, Japan, and Korea as well as some western countries. Although the 5-year survival rate for early gastric cancer is greater than 90% after surgery, the prognosis for advanced gastric cancer that cannot be surgically treated is poor, with a 5-year survival rate of less than 10%. Approximately two-thirds of patients with gastric cancer are diagnosed at an advanced stage in China and they can only be treated with chemotherapy and/or radiotherapy. Although various chemotherapeutic agents, such as epirubicin, fluorouracil, docetaxel and cisplatin, have been used for advanced gastric cancer and survival rates have improved, the efficacy of chemotherapy is limited. To date, there is still no standard chemotherapeutic regimen for advanced gastric cancer. The development of more efficacious therapies remains a great challenge for the treatment of this disease.

Bufalin is one of the major active components of Chan Su, a traditional Chinese medicine obtained from the skin and parotid venom glands of toad [2]. In China, Chan Su has been widely used as a traditional Chinese medicine for the treatment of some cancers and some studies have shown that bufalin induced apoptosis in some human leukemia and solid cancer cell lines [3–5]. Watabe *et al.* [6] reported that persistent activation of mitogen-activated protein kinase/extracellular-regulated protein kinases (ERK) was one of the signaling transduction pathways in bufalin-induced apoptosis in leukemia U937 cells. We have reported that bufalin downregulated Bcl-2, survivin and WT1, activated PKCβII and caspase-3, and released Smac/DIABLO from mitochondria during apoptosis in leukemia cells [7–9]. The exact mechanism of bufalin-induced apoptosis is, however, still not clear.

Many studies have demonstrated that the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays a critical role in controlling the balance between cell survival and apoptosis [10–12]. Activated Akt phosphorylates several key downstream effectors such as Bad [13], procaspase-9 [14], the transcription factor FKHRL1 [10], and I-κB kinase [15], thus promoting survival and blocking apoptosis in some cancer cells. Increasing evidence shows that many cytotoxic drugs induce apoptosis in cancer cells by inhibiting the PI3K/Akt pathway [16–18]. It, however, remains unknown whether bufalin affects the PI3K/Akt pathway during apoptosis of tumor cells, especially gastric cancer cells.

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In this study, we demonstrated that bufalin transiently activates and then inhibits the PI3K/Akt signaling pathway, accompanied by the upregulation of Casitas B-lineage lymphoma (Cbl)-b and c-Cbl, upstream regulators of PI3K, during apoptosis of gastric cancer cells.

Materials and methods

Reagents and antibodies

Anti-caspase-3, anti-Bax, anti-Bcl-2, anti-tubulin and anti-Cbl-b antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Anti-phospho-Akt (Ser-473) and anti-Akt antibodies were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Anti-c-Cbl antibody was purchased from Transduction Laboratories (Lexington, Kentucky, USA). Bufalin and LY294002 were purchased from Sigma-Aldrich (St Louis, Missouri, USA). PD98059 was purchased from Promega (Madison, Wisconsin, USA).

Cell culture

Human gastric adenocarcinoma MGC803 cells obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C under an atmosphere of 95% air and 5% CO_2. Cells were routinely subcultured every 2–3 days and cell samples used were all in the logarithmic growth phase.

3-(4, 5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

The effect of bufalin on MGC803 cell proliferation was measured using the 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded at 5×10^3 cells/well in 96-well plates and incubated overnight, and then different concentrations (1–160 nmol/l) of bufalin were added and further incubated for the indicated time. Thereafter, 20 µl of MTT solution (5 mg/ml) was added to each well and the cells were incubated for another 4 h at 37°C. After the removal of the culture medium, the cells were lysed in 150 µl of dimethylsulfoxide and then the optical density (OD) was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, California, USA). The following formula was used: cell viability = (OD of the experimental sample/OD of the control group) × 100%.

Cell cycle phase analysis

Phase distributions of the cell cycle and hypodiploid DNA were determined by flow cytometry. Cells were exposed to 20 and 80 nmol/l bufalin for 24 h and then collected and washed twice with phosphate-buffered saline (PBS). After fixing in ice-cold 70% ethanol for 12 h, the samples were washed twice with PBS and then incubated with 20 µg/ml RNase A and 10 µg/ml propidium iodide for 30 min in the dark. Finally, the samples were evaluated by flow cytometry and the data were analyzed using CellQuest software (Becton Dickinson, San Jose, California, USA).

Morphology staining and mitotic index determination

Cells were seeded at 2.5×10^4 cells/well in 6-well plates and incubated overnight, and then exposed to 20 and 80 nmol/l bufalin for 24 h. Morphological analysis was performed by cytospin preparation using the Wright–Giemsa stain (Sigma). The slides were observed and photographed under a light microscope, and a minimum of 1000 cells were counted on each slide to determine the mitotic index.

Western blot analysis

Cells were washed twice with ice-cold PBS and solubilized in 1% Triton lysis buffer (1% Triton X-100, 50 mmol/l Tris–Cl, pH 7.4, 150 mmol/l NaCl, 10 mmol/l EDTA, 100 mmol/l NaF, 1 mmol/l Na_3VO_4, 1 mmol/l phenylmethanesulphonylfluoride, and 2 µg/ml aprotinin) on ice, then quantified by the Lowry method. Samples of 50 µg of cell lysate were separated by SDS–polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Immoblin-P, Millipore, Bedford, Massachusetts, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline Tween 20 (TBST) buffer (10 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, and 0.1% Tween 20) at room temperature for 2 h and incubated at 4°C overnight with the indicated primary antibodies. After washing with TBST buffer, membranes were reacted with the appropriate horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. After extensive washing with TBST buffer, proteins were visualized using an enhanced chemiluminescence reagent (SuperSignal Western Pico Chemilunescent Substrate; Pierce, Rockford, Illinois, USA).

Statistical analysis

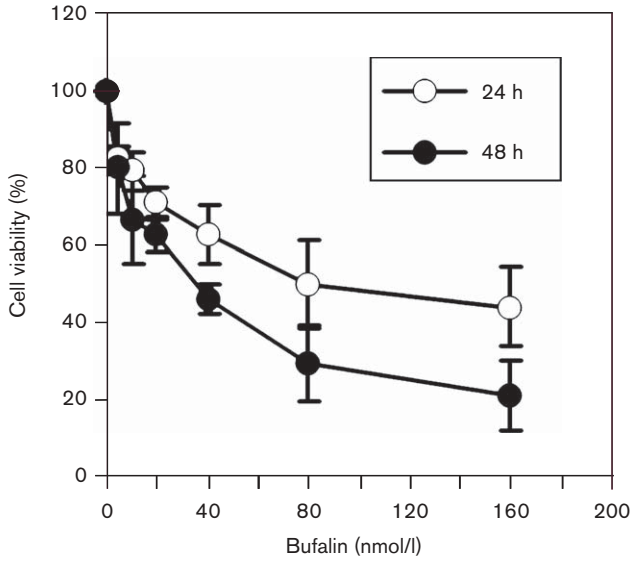
Data are expressed as mean ± SD. Differences between two groups were evaluated by Student's *t*-test. Comparisons among three or more groups were evaluated by one-way analysis of variance followed by the Studentized Newman Keuls test. A P value of less than 0.05 was considered to be statistically significant.

Results

Bufalin inhibits MGC803 cell proliferation

To verify whether bufalin inhibits proliferation of gastric cancer cells, MGC803 cells were exposed to the concentrations of bufalin indicated for 24 and 48 h. MTT assays revealed that bufalin inhibited MGC803 cell proliferation in a dose-dependent and time-dependent manner (Fig. 1). The concentration inhibiting 50% cell growth at 24 and 48 h was 96.03 ± 7.58 and 29.72 ± 1.57 nmol/l, respectively.

Fig. 1



Bufalin inhibited MGC803 cell proliferation. MGC803 cells were exposed to bufalin (1–160 nmol/l) for 24 and 48 h and cell viability was then determined by 3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Data are the mean ± SD of at least three independent experiments performed in triplicate.

Bufalin induces M-phase cell cycle arrest and apoptosis in MGC803 cells

As shown in Fig. 2a, an increase in G_2/M phase was observed after cell exposure to 20 nmol/l bufalin for 24 h. The percentage of cells in G_2/M phase increased from 28.58 ± 3.34 to $41.13 \pm 4.85\%$ ($P = 0.017$). Furthermore, morphological analysis demonstrated that most of the cells were in a binucleate state without cell division, and some were in metaphase after exposure to 20 nmol/l bufalin for 24 h (Fig. 2b). The mitotic index of treated cells was higher than that of untreated control cells (15.97 ± 1.96 vs. $3.57 \pm 0.32\%$; $P = 0.000$), indicating that bufalin induced M-phase cell cycle arrest in MGC803 cells. When the cells were exposed to 80 nmol/l bufalin for 24 h, obvious apoptosis (sub-G1 peak) occurred (Fig. 2a) and the percentage of apoptotic cells increased from 1.35 ± 0.47 to $18.76 \pm 2.42\%$ ($P = 0.000$). Morphological analysis revealed that apoptotic cells became rounded in shape and their nuclei exhibited a fragmented morphology, forming apoptotic bodies (Fig. 2b). These results clearly show that bufalin induced M-phase cell cycle arrest at low concentrations, but induced apoptosis at high concentrations in MGC803 cells.

To identify the mechanism of bufalin-induced apoptosis in MGC803 cells, we investigated the levels of proteins related to apoptosis. Bufalin caused cleavage of procaspase-3, increased expression of the proapoptotic molecule Bax and slightly decreased that of the antiapoptotic molecule Bcl-2 (Fig. 2c). After exposure to 20 and 80 nmol/l bufalin for 24 h, the Bax/Bcl-2 ratio was 1.69-fold and 2.20-fold greater than that of the untreated control group, respectively. These results suggest that bufalin-induced apoptosis is associated with activation of caspase-3, upregulation of Bax and downregulation of Bcl-2.

Inhibition of PI3K/Akt, but not ERK, enhances bufalin-induced apoptosis

To determine whether the PI3K/Akt signaling pathway is involved in bufalin-induced apoptosis, we investigated Akt activation in MGC803 cells after treatment with 80 nmol/l bufalin for 1–16 h. As shown in Fig. 3a, bufalin did not affect total Akt protein levels. Akt phosphorylation, however, increased slightly at 1 and 8 h and then was completely inhibited at 16 h compared with untreated control cells. Pretreatment with 25 μmol/l LY294002, a specific inhibitor of PI3K, for 1 h before exposure to 80 nmol/l bufalin for 1 and 8 h completely blocked Akt activation (Fig. 3b). We next investigated whether the presence of LY294002 could affect bufalin-induced apoptosis in MGC803 cells. As shown in Fig. 3c and d, LY294002 alone had little effect on apoptosis. However, when cells were pretreated with 25 μmol/l LY294002 for 1 h and then exposed to 80 nmol/l bufalin for 8 h, the percentage of apoptotic cells increased from 4.61 ± 0.80 to $12.67 \pm 1.22\%$ ($P = 0.000$). In contrast, pretreatment of MGC803 cells with the ERK-specific inhibitor, PD98059 (20 μmol/l), for 1 h had no significant effect on bufalin-induced apoptosis. Taken together, these results indicate that the PI3K/Akt signaling pathway, but not the ERK signaling pathway, plays a crucial role in regulating bufalin-induced apoptosis in MGC803 cells.

Bufalin upregulates the expression of Cbl-b and c-Cbl

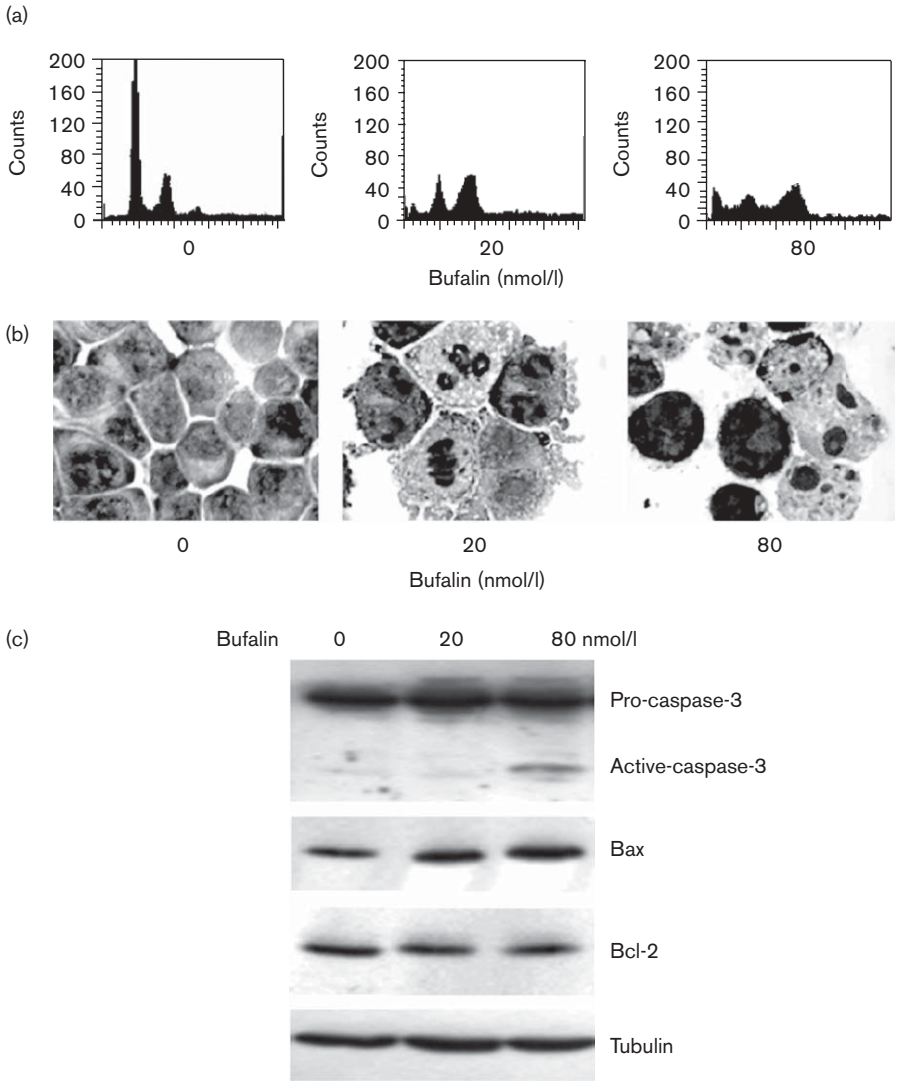
To investigate whether inhibition of Akt activation by bufalin was correlated with the Cbl family of ubiquitin ligases, we investigated the expression of Cbl-b and c-Cbl. As shown in Fig. 4, after treatment with 80 nmol/l bufalin for 16 h, the expression levels of Cbl-b and c-Cbl were 2.83-fold and 2.01-fold greater than those in the untreated control group, respectively.

Discussion

Earlier studies have shown that bufalin can inhibit cell proliferation and induce apoptosis in leukemia [3], osteosarcoma [4], and prostate cancer cells [5]. These results show that bufalin inhibited proliferation in a dose-dependent and time-dependent manner and induced M-phase cell cycle arrest and apoptosis in MGC803 cells, indicating that growth inhibition by bufalin may be because of apoptosis and M-phase arrest of cell cycle progression.

It has been reported that bufalin can induce apoptosis by the activation of AP-1 [19], the c-Jun N-terminal protein kinase [19,20], Rac1 [20], cdc2 kinase, and casein

Fig. 2

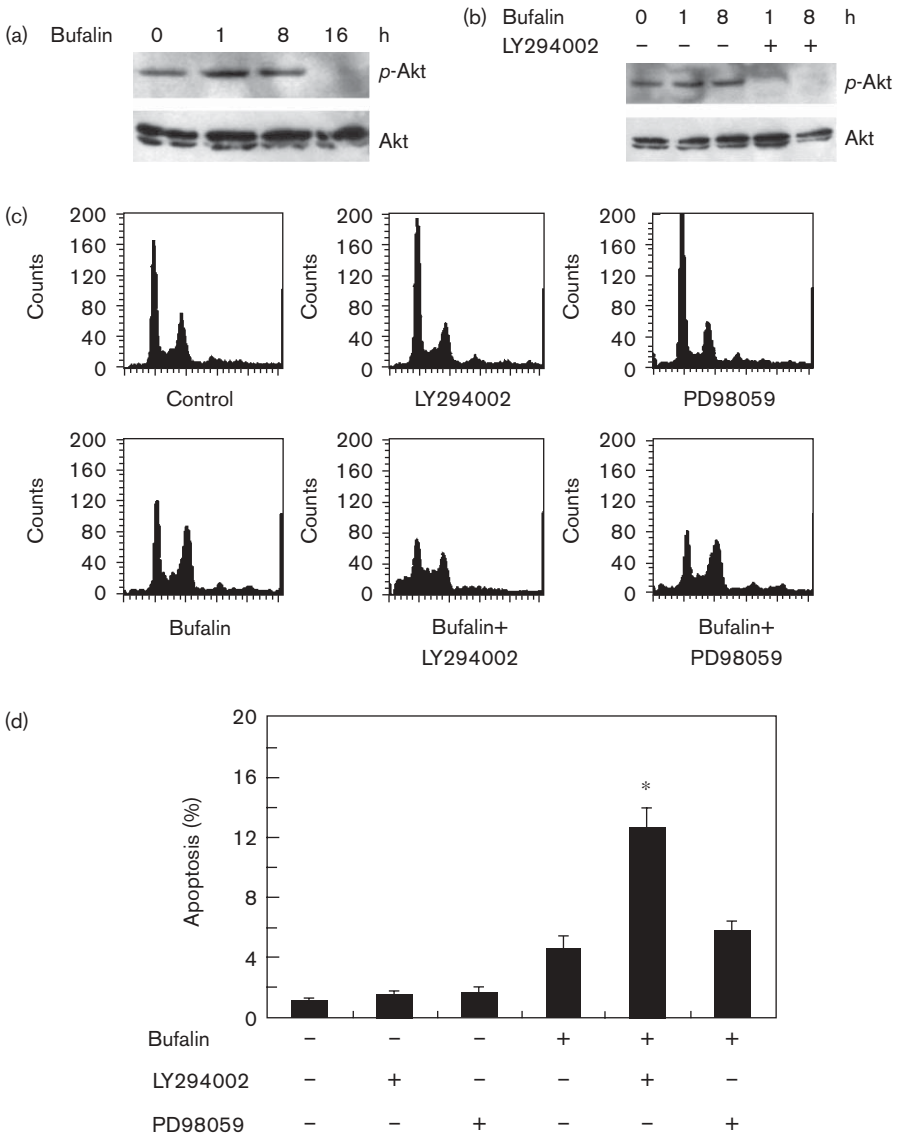


Bufalin induced M-phase cell cycle arrest and apoptosis in MGC803 cells. Cells were exposed to 20 and 80 nmol/l bufalin for 24 h and then the cell cycle was analyzed by flow cytometry after staining with propidium iodide (a). Cell morphological changes were determined by light microscopy (magnification $\times$1000) after Wright–Giemsa staining (b). The expression of caspase-3, Bax, and Bcl-2 proteins was analyzed by western blotting. Tubulin was used as the internal control (c). Data are representative of one of three independent experiments.

kinase II [21], as well as by inhibition of Bcl-2, c-myc [3], protein kinase A, and protein kinase C [21] in leukemia cells. These data show that bufalin-induced apoptosis was correlated with upregulation of Bax, downregulation of Bcl-2 and activation of caspase-3 in gastric cancer cells. These results indicate that the mechanism of bufalin-induced apoptosis is not the same in different cell lines.

Cell survival is dependent on a balance between survival and death signals. PI3K/Akt and mitogen-activated protein kinase/ERK signaling pathways are two important signaling transduction systems in the cell. Activation of Akt and ERK promotes cell proliferation and inhibits apoptosis through a number of downstream targets. It has been reported that Akt and ERK are highly expressed and persistently activated in most cancers [12,22], and inhibition of PI3K/Akt and ERK signaling pathways can induce apoptosis in some human cancer cell lines [23–26]. In this study, we observed that Akt was transiently activated by bufalin and that inhibition of the PI3K/Akt signaling pathway by LY294002 significantly enhanced bufalin-induced apoptosis at 8 h. PD98059, a specific inhibitor of ERK, however, had no significant effect on bufalin-induced apoptosis, indicating that the PI3K/Akt pathway, but not the ERK pathway, may play a critical role in the survival of MGC803 cells after exposure to bufalin. In contrast, bufalin inhibited Akt activation completely at 16 h, which might contribute to bufalin-induced apoptosis.

Fig. 3

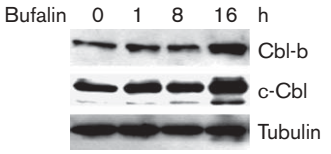


Inhibition of phosphatidylinositol 3-kinase/Akt, but not extracellular-regulated protein kinases, enhanced bufalin-induced apoptosis. (a and b) MGC803 cells were exposed to 80 nmol/l bufalin in the absence or presence of 25 μmol/l LY294002 and the cell lysates were analyzed by western blotting using antibodies specific for Akt and *p*-Akt. To control for loading inequities, the relative amount of Akt protein in each sample is shown in the lower panel. (c) Cells were exposed to 80 nmol/l bufalin for 8 h in the absence or presence of 25 μmol/l LY294002 or 20 μmol/l PD98059 and the cell cycle was analyzed by flow cytometry after staining with propidium iodide. A representative experiment that was reproduced three times is shown. (d) The percentage of apoptotic cells was analyzed by flow cytometry. *$P<0.05$ vs. bufalin alone. Data are the mean ± SD of three independent experiments.

Regarding the role of ERK in bufalin-induced apoptosis, Watabe *et al.* [6] reported that excessive activation of the ERK cascade was necessary for bufalin-induced apoptosis in U937 cells. Our previous study demonstrated that ERK inhibition enhanced bufalin-induced apoptosis in K562 cells [27]. These discrepancies may be attributed to differences in the cell systems used and suggest that the effects of bufalin are cell-type specific.

The PI3K/Akt pathway is regulated by various factors and recent studies have suggested that ubiquitin ligases Cbl-b and c-Cbl, which ubiquitinylate some important signaling molecules, could negatively regulate PI3K [28–31]. Furthermore, the p85 regulatory subunit of PI3K has been identified as a substrate of Cbl-b and c-Cbl, and Cbl proteins direct PI3K ubiquitination [32,33]. To investigate whether inhibition of Akt activation by bufalin was correlated with Cbl protein levels, we determined the expression of Cbl-b and c-Cbl. These Cbl proteins were significantly upregulated after cell exposure to bufalin for 16 h, indicating that they might contribute to inhibition of the PI3K/Akt signaling pathway

Fig. 4



Bufalin upregulated the expression of Casitas B-lineage lymphoma (Cbl)-b and c-Cbl. Cells were exposed to 80 nmol/l bufalin for 1–16 h, and expression of Cbl-b and c-Cbl proteins was analyzed by western blotting. Tubulin was used as the internal control.

through ubiquitination of PI3K. However, the mechanism of bufalin-mediated upregulation of Cbl proteins and the role of Cbl in bufalin-induced apoptosis require further investigation.

In conclusion, this study showed that bufalin possibly induces apoptosis by inhibiting the PI3K/Akt signaling pathway in gastric cancer cells. Moreover, Cbl-b and c-Cbl might be involved in the inhibition of the PI3K/Akt pathway.

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