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Licorice and Licochalcone-A Induce Autophagy in LNCaP Prostate Cancer Cells by Suppression of Bcl-2 Expression and the mTOR Pathway

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Licorice is a common Chinese medicinal herb with antitumor activity. Some components in licorice root have been shown to induce cell cycle arrest or apoptosis in cancer cells. This paper demonstrates for the first time that licorice *Glycyrrhiza glabra* and its component licochalcone-A (LA) can induce autophagy in addition to apoptosis in human LNCaP prostate cancer cells. Exposure of cells to licorice or LA resulted in several confirmed characteristics of autophagy, including the appearance of autophagic vacuoles revealed by monodansylcadaverine (MDC) staining, formation of acidic vesicular organelles (AVOs), and autophagosome membrane association of microtubule-associated protein 1 light chain 3 (LC3) characterized by cleavage of LC3 and its punctuate redistribution, as well as ultrastructural observation of autophagic vacuoles by transmission electron microscopy. Autophagy induction was accompanied by down-regulation of Bcl-2 and inhibition of the mammalian target of rapamycin (mTOR) pathway. In summary, licorice can induce caspase-dependent and autophagy-related cell death in LNCaP cells.

KEYWORDS: Licorice; licochalcone-A; autophagy; cell death; Bcl-2; mTOR; LNCaP cells

INTRODUCTION

Prostate cancer is the most common malignant tumor in male adults in the Western world, which represents a major cause of cancer-related morbidity and mortality. Botanic mixtures are widely used by patients with prostate cancer. Licorice, the root of the plant *Glycyrrhiza glabra*, comprises flavonoids and pentacyclic triterpene saponin as major constituents, which include licochalcone-A (LA), liquiritin, liquiritigenin, isoliquiritigenin, liquiritin apioside, glycyrrhizin, and glycyrrhizic acid (*1*, *2*). Previous studies have demonstrated that licorice root extract has many potent biological effects, including antitumor activity (*3*−*5*). A water extract of licorice was found to inhibit angiogenesis (*6*). It has been shown that licorice root and some of its components, such as LA and isoliquiritigenin, induce cell cycle arrest or apoptosis in a variety of cancer cells (*1*, *5*, *7*−*10*).

Autophagy is a term used to describe the process of protein recycling, typically observed in hepatocytes after amino acid deprivation (*11*). It is characterized by the observation of double- or multiple-membrane cytoplasmic vacuoles devouring bulk cytoplasm and/or cytoplasmic organelles such as mitochondria and endoplasmic reticulum in type II autophagy-related cell death (*12*). Bcl-2, which is an integral membrane protein located mainly on the outer membrane of mitochondria, can prevent apoptosis by blocking cytochrome *c* release from mitochondria (*13*). Apoptotic cell death is triggered by down-regulation of Bcl-2 and cytochrome *c* release, and consequently the caspase cascade is activated to promote apoptosis (*13*). LA, which possesses antitumor activities (*1*, *10*, *14*), can modify the Bcl-2 level (*10*). A novel polyphenol molecule isolated from licorice root was also shown to induce Bcl-2 phosphorylation and apoptosis in prostate and breast cancer cells (*5*). Bcl-2 functions not only as an antiapoptotic protein but also as an antiautophagic protein through its inhibitory interaction with beclin 1 (*15*). Beclin 1, a mammalian homologue of yeast *Atg6*/*Vps30*, is required for the induction of autophagy in response to nitrogen deprivation (*16*). Furthermore, the Akt/mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase (p70S6K) pathway can negatively regulate autophagy in response to starvation in mammalian cells (*17*).

We report here a novel function of licorice and its component LA to induce autophagy, in addition to their apoptotic function. Furthermore, licorice can induce mitochondria-related apoptosis and autophagy-related cell death in LNCaP cells.

MATERIALS AND METHODS

Cells. Human androgen-dependent LNCaP prostate cancer cells were grown in RPMI 1640 containing 10% fetal bovine serum, 2 mM

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L-glutamine, nonessential amino acids, and 50 μg/mL gentamicin at 37 °C in a humidified atmosphere of 5% CO_2. LNCaP cells were transfected with pcDNA3-Bcl-2 (*18*) and pcDNA3 (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 (Invitrogen) to generate Bcl-2-overexpressing and vector-control LNCaP cells, respectively.

Preparation of Herbal Extracts. Licorice (*G. glabra* L.) powder obtained from Chuang Song Zong Pharmaceutical Co. (Kaohsiung, Taiwan) was dissolved in 70% pyrogen-free ethanol (*14*) at a concentration of 320 mg/mL. The mixture was then vortexed rigorously for 2 min followed by vigorous shaking for 1 h at 37 °C. After being centrifuged at 3000*g* for 10 min, the extract was collected and stored at −20 °C. The extract (1 mL) was lyophilized to dryness, weighed, and redissolved to 1 mL of 70% ethanol. The final concentration of the licorice extract was 114 mg/mL (*19*, *20*). The licorice component LA (Calbiochem, La Jolla, CA) was dissolved in DMSO as the stock (10 mM) and stored at −20 °C until use.

Cell Viability Assay. LNCaP cells were treated with various concentrations of licorice or LA at different time intervals. The CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI) based on the release of lactate dehydrogenase (LDH) was used to measure the cytotoxicity of licorice on LNCaP cells according to the manufacturer's instructions (*21*). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVADfmk, R&D Systems, Minneapolis, MN), monobromobimane (MBB, Sigma Chemical Co., St. Louis, MO), and bafilomycin A (BafA1, Sigma) were included in some experiments during the treatment of licorice.

Cell Cycle and Apoptosis Analysis. For cell cycle analysis, LNCaP cells were treated with licorice (228 μg/mL) alone or in the presence of zVADfmk (50 μM), MBB (1 μM), or BafA1 (10 nM) for 24 h. LNCaP cells treated with etoposide (30 μg/mL) served as the positive control for apoptotic cell death. Total cells were harvested, fixed, and permeabilized in chilled 70% ethanol overnight at −20 °C. After centrifugation, cells were washed and resuspended in PBS containing Triton X-100 (0.1%), RNase (0.2 mg/mL), and propidium iodide (PI, 20 μg/mL) for determining cell cycle profiles. Samples were analyzed for DNA content with the FACScan flow cytometer (BD Biosciences, San Diego, CA), and relative cell cycle distribution was analyzed using the CellQuest software (Verity Software House, Topsham, ME).

A DeadEnd colorimetric terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit (Promega) was used to detect apoptotic cells according to the manufacturer's instructions. Briefly, LNCaP cells were treated with licorice (228 μg/mL) for 24 h, fixed with 4% paraformaldehyde in PBS, washed once with PBS, permeabilized with 0.2% Triton X-100, and then washed twice in PBS. TUNEL-positive cells with dark brown staining were observed under a light microscope.

Measurement of Mitochondrial Membrane Potential (MMP). MMP was measured by DiOC6(3) uptake as previously described (*22*). Briefly, LNCaP cells were treated for 24 h with various concentrations of licorice and then stained with 40 nM DiOC6(3) (Molecular Probes, Eugene, OR) at 37 °C for 30 min. MMP was determined by measuring the retention of DiOC6(3) with a FACSCalibur flow cytometer (BD Biosciences). Data were sorted by analyzing 20000 events using the CellQuest software. The fraction of cells showing low MMP was measured by flow cytometry.

Visualization and Analysis of Intracellular Autophagic Vacuoles. LNCaP cells treated with licorice or LA as indicated concentrations (*1*) for 24 h were stained at room temperature for 30 min with 50 mM monodansylcadaverine (MDC, Sigma), an autofluorescent base capable of accumulating in autophagic vacuoles (*23*). The autophagic vacuoles were visualized by a fluorescence microscope with a digital camera. The autophagic index was determined as the percentage of MDC-labeled cells of 100 cells from each sample (*24*). Autophagy can also be characterized by the development of acidic vesicular organelles (AVOs) (*25*, *26*). LNCaP cells treated with licorice (228 μg/mL) for 24 h were stained with acridine orange (1 μg/mL, Sigma) for 20 min, and the presence of AVOs was examined by fluorescence microscopy (*26*). Flow cytometry was also used to quantify AVOs. In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red (*26*). The intensity of the red fluorescence is proportional to the degree of acidity. Therefore, the volume of the cellular acidic compartment can be quantified. The acridine orange-stained cells were collected and analyzed with a FACSCalibur flow cytometer using FL3-H mode (> 650 mm) to value the bright red fluorescence and FL1-H mode (500−550 nm) to value the green fluorescence as described previously (*26*). Furthermore, the green fluorescent protein (GFP)-fused microtubule-associated protein 1 light chain 3 (LC3) was used to detect autophagy as described previously (*27*). LNCaP cells were transfected with 2 μg of the GFP-LC3 expression plasmid using Lipofectamine 2000. After 24 h, the transfected cells were treated with licorice for 24 h, and the distribution and fluorescence of GFP-LC3 were visualized by fluorescence microscopy.

Transmission Electron Microscopy (TEM). LNCaP cells treated with licorice or LA for 24 h were fixed in situ for 10 min in 50% Karnovsky fixative. Cells were collected and centrifuged at 1500*g* for 5 min. The pellet was washed and stored in 70% Karnovsky fixative at 4 °C until embedding and then analyzed by TEM. The ultrathin sections were viewed with a Hitachi 7000 electron microscope (Tokyo, Japan). Twenty cell sections were analyzed, and the percentage of the cell area taken up by autophagic vacuoles was determined with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) (*28*).

Immunoblot Analysis. Equal protein amounts of total cell lysates (20−50 μg) from LNCaP cells following various treatments were subjected to immunoblot analysis as described previously (*24*). Immunoblotting was used to assess the processing of LC3-I to LC3-II, a marker for autophagy (*29*), using anti-LC3 antibody (MBL, Nagoya, Japan). The primary antibodies against beclin 1 (clone 20, BD Biosciences), Bcl-2 (clone 100, Santa Cruz Biotechnology, Santa Cruz, CA), phospho(ser473)-Akt (Cell Signaling Technology, Beverly, MA), phospho(ser248)-mTOR (Cell Signaling), mTOR (Cell Signaling), phospho(thr389)-p70S6K (Cell Signaling), p70S6K (Cell Signaling), and β-actin (clone AC-15, Sigma), as well as horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and anti-mouse IgG (Jackson), were used for immunoblot analysis.

Statistical Analysis. Data were expressed as mean ± SD. For the statistical analysis, Student's *t* test was used. Values of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Licorice Induced Cell Cycle Arrest and Apoptosis in LNCaP Cells. To confirm previous results showing induction of cell cycle arrest or apoptosis in cancer cell lines by licorice root and its components (*1*, *5*, *7*−*10*), we first examined whether licorice extract could induce cell death and apoptosis in prostate cancer cells. **Figure 1A** shows that licorice dose- and time-dependently induced cell death in LNCaP cells, as determined by the cell viability assay (LDH release assay), which measures the release of the intracellular enzyme LDH upon damage of the plasma membrane (*21*). Next, cell cycle was analyzed by flow cytometry in LNCaP cells following treatment with licorice during a period of 24 h. **Figure 1B** shows that licorice arrested the cells in the G2/M phase and increased the sub-G1-phase cells. Because apoptotic cells have a lower DNA content than nonapoptotic cells, a prominent sub-G1 peak that appeared in the DNA content of the cells after exposure to licorice for 24 h suggested that the treated cells might undergo apoptosis. We next applied the TUNEL assay to confirm the apoptotic cell death induced by licorice in LNCaP cells. Whereas the number of TUNEL-positive, dark brown cells was minimal in untreated cells, it was increased after licorice treatment (**Figure 1C**). Notably, some dead cells appeared to be TUNEL-negative, which stained blue in licorice-treated cells, suggesting that pathways other than apoptosis may exist in licorice-induced cell death in LNCaP cells (*30*).

Licorice Induced Mitochondria-Dependent Cell Death. The mitochondrion plays a central role in determining cell survival or death in response to diverse stimuli (*31*). Loss of MMP is associated with dysfunction of mitochondria, which can be detected in both apoptotic and necrotic cell deaths (*32*).

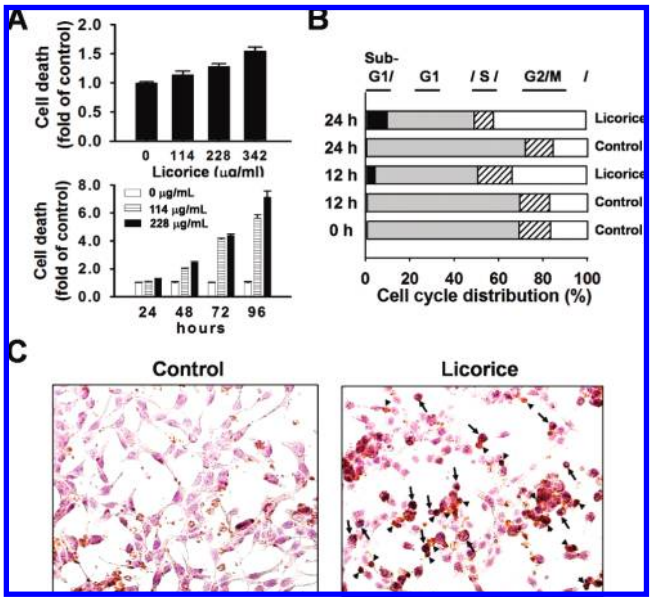

Figure 1. Licorice induced cell cycle arrest and apoptosis in LNCaP cells. (**A**) Licorice induced cell death in a dose- and time-dependent manner. Cells were treated with indicated concentrations of licorice for 24 h (upper panel) or for various periods of time (lower panel), and their survival was determined by the cell viability assay (LDH release assay). Data are mean ± SD ($n = 5$). (**B**) Licorice arrested the cells in the G2/M phase and increased the sub-G1-phase cells. For the determination of cell cycle distribution, the DNA content of LNCaP cells was analyzed by PI staining and flow cytometry at 12 and 24 h after treatment with licorice (228 μg/mL). (**C**) Licorice induced apoptotic and nonapoptotic cell death. Cells treated with licorice (228 μg/mL) for 24 h were stained by TUNEL for detection of apoptosis. Arrowheads denote TUNEL-positive cells, which appeared dark brown, whereas arrows denote TUNEL-negative dead cells, which were round and appeared blue.

To examine whether the mitochondria-dependent pathway for apoptosis was related to licorice-induced cell death, we measured the MMP change by quantification of DiOC6(3) fluorescence. The percentage of low-DiOC6(3) fluorescence-gated cells was increased dose-dependently following licorice treatment (**Figure 2A**), suggesting that dissipation of the MMP was associated with licorice-induced cell death. To this end, isoliquiritigenin, a component of licorice, was also shown to induce cell death by modulation of the MMP in human DU145 prostate cancer cells (*33*).

Because disruption of the MMP is associated with both caspase-dependent and caspase-independent apoptotic cell death, we analyzed cellular DNA content of LNCaP cells treated with licorice by flow cytometry to determine the percentages of cells undergoing apoptosis (sub-G1%). We also applied MBB, which is an agent capable of preventing apoptosis-inducing factor (AIF)-associated caspase-independent apoptosis (*31*) and the pan-caspase inhibitor zVADfmk to further gain insight into the mechanism of licorice-induced cell death. Whereas zVADfmk completely abolished licorice-induced apoptotic cell death, MBB treatment did not have any effect on cell apoptosis (**Figure 2B**). These results suggest that the apoptotic cell death induced by licorice was caspase-dependent. Furthermore, cell death induced by licorice could be abrogated to a larger extent by zVADfmk, but not by MBB treatment, as determined by the cell viability assay (LDH release assay) (**Figure 2C**). Notably, zVADfmk could not completely prevent licorice-induced cell death. Taken together, these results suggest that a nonapoptotic cell death pathway may also exist in licorice-treated LNCaP cells.

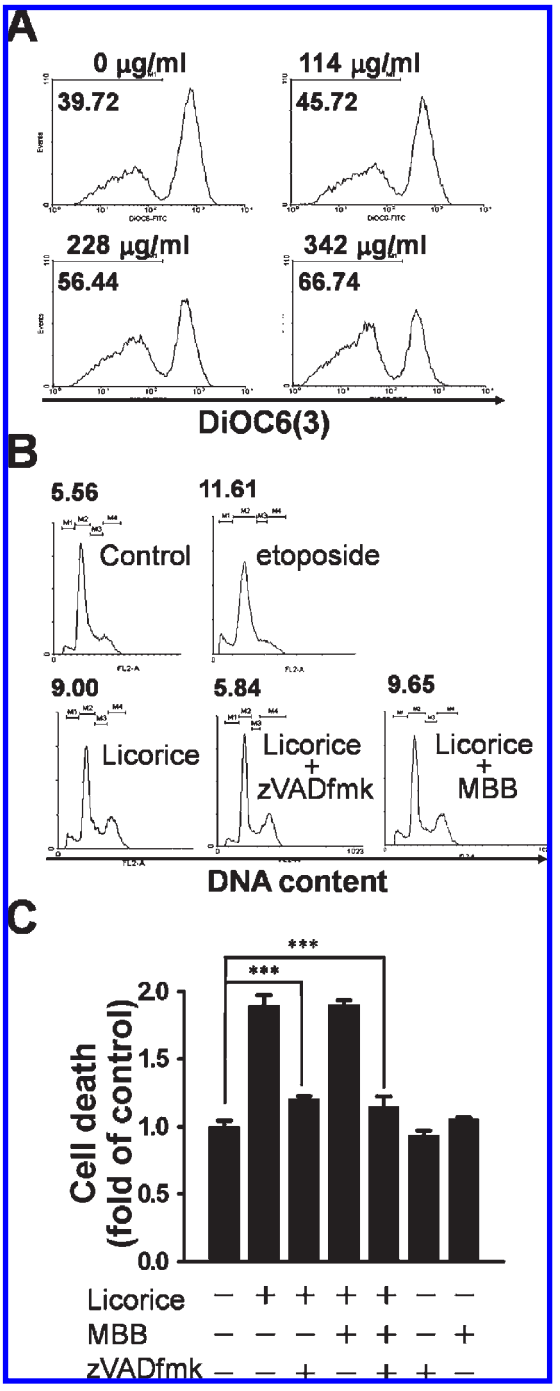

Figure 2. Licorice induced mitochondria-dependent cell death in LNCaP cells. (**A**) Licorice reduced MMP. Cells treated with the indicated concentrations of licorice for 24 h were stained with DiOC6(3) for measurement of the MMP by flow cytometry. Numbers indicate the percentage of low-DiOC6(3) fluorescence-gated cells. (**B**) The pan-caspase inhibitor zVADfmk, but not MBB, which inhibits caspase-independent apoptosis, completely abolished licorice-induced apoptotic cell death measured by the sub-G1 cell population. Cells were treated with licorice (228 μg/mL) alone or in the presence of zVADfmk (50 μM) or MBB (1 μM) for 24 h. Cells treated with etoposide (30 μg/mL) for 24 h served as the positive control for apoptotic cell death. Cellular DNA content was analyzed by PI staining and flow cytometry. Numbers indicate the percentages of sub-G1 cell population (apoptotic cells). (**C**) Cell death induced by licorice could not be completely abrogated by zVADfmk treatment, as determined by the cell viability assay (LDH release assay). Cells were treated as described in **B**. Cell death is presented as the ratio of the LDH released from the licorice-treated cells to that from the untreated control cells. Values are mean ± SD ($n = 5$). ***, $P < 0.001$.

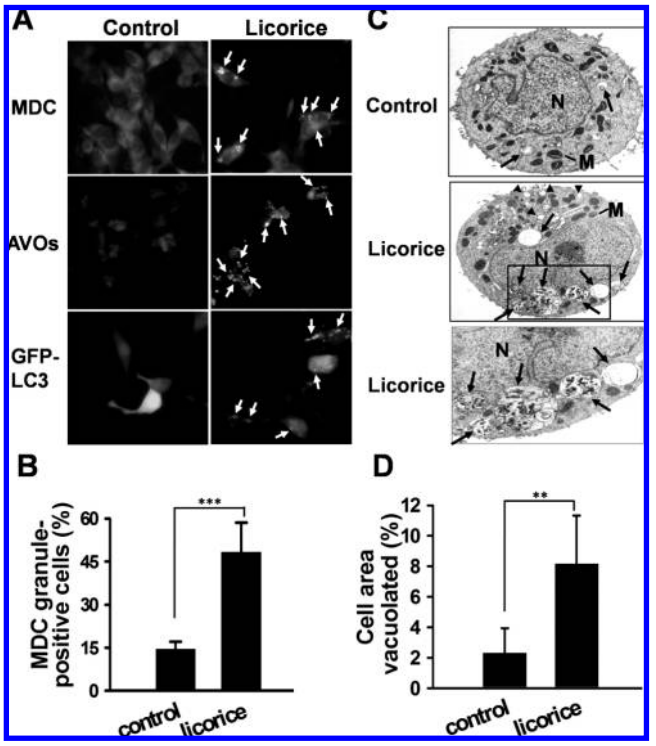

Figure 3. Licorice induced autophagy in LNCaP cells. Cells were treated with licorice (228 μg/mL) for 24 h and assessed for autophagy formation by different methodologies. (**A**) Detections of autophagic vacuoles and AVOs by MDC staining (upper two panels) and acridine orange staining (middle two panels), respectively, in licorice-treated cells, and observation of typical punctate pattern of GFP-LC3 in GFP-LC3-transfected cells treated with licorice (lower two panels). Note the presences of autophagic vacuoles (upper right panel), AVOs (middle right panel), and punctate fluorescence of GFP-LC3 (lower right panel) in the cytoplasm of licorice-treated cells denoted by arrows. Similar results were observed in at least three independent experiments. (**B**) Quantification of autophagic vacuoles in licorice-treated cells (see arrows in the upper right panel in **A**). A minimum of 100 cells was counted for each sample. Values are means ± SD (n = 3), which were consistent in three independent experiments. ***, $P < 0.001$. (**C**) Licorice-treated cells (middle panel) and their control counterparts (upper panel) subjected to TEM (5000× magnification). The lower panel shows the magnified image (10000× magnification) of the area indicated by the box in the middle panel. Note the abundance of autophagic vacuoles (arrows) and empty vacuoles (arrowheads) in licorice-treated cells, which were rarely seen in the control cells. N, nucleus. M, mitochondrion. (**D**) Morphometric analysis of cells treated with or without licorice. Ratio of total area of autophagic vacuoles to the total cytoplasmic area is shown. Values are means ± SD (n = 20), which were consistent in two independent experiments. **, $P < 0.01$.

Licorice Induced Autophagy. Many anticancer drugs induce apoptosis. However, some chemotherapeutic agents were reported to induce autophagy-related cell death in certain cancer cell lines. For instance, paclitaxel induced autophagy-related cell death as well as apoptotic cell death in some cancer cells (*34*). As our results revealed that licorice induced nonapoptotic cell death as well as caspase-dependent apoptotic cell death in LNCaP cells, we sought to investigate whether licorice also induced autophagy-related cell death in this cell line. The fluorescent dye MDC is an in vivo specific marker for autophagic vacuoles (*23*). When MDC-stained cells are visualized by fluorescence microscopy, MDC accumulates in mature autophagic vacuoles, such as autophagolysosomes, with distinct dot-like structures distributing within the cytoplasm or localizing in the perinuclear regions. In licorice-treated LNCaP cells, the accumulation of MDC was noticeable as revealed by punctate MDC fluorescence (**Figure 3A**). The percentage of cells with MDC accumulation was significantly higher in licorice-treated cells than in the control cells (**Figure 3B**). In addition to MDC incorporation, autophagy can also be characterized by the development of AVOs (*25*, *26*), which can be detected and measured by vital staining of acridine orange. Acridine orange is a lysosomo-tropic agent, which moves freely across biological membranes when uncharged, whereas its protonated form accumulates in acidic compartments, where it forms aggregates with bright red fluorescence (*35*). Vital staining of LNCaP cells with acridine orange revealed concentrated dye in the vesicles that fluoresced bright red, indicating the appearance of AVOs (**Figure 3A**). In marked contrast, the majority of untreated cells exhibited diffuse green fluorescence. During the autophagic process, LC3 is concentrated in autophagosome membranes, and the punctate fluorescence produced by GFP-LC3 can be used as a good indicator of autophagy (*27*). We also transfected the GFP-LC3 expression plasmid into LNCaP cells to visualize autophagy. As shown in **Figure 3A**, untreated LNCaP cells showed diffuse cytoplasmic distribution of green fluorescence, whereas punctate fluorescence of GFP-LC3, characteristic of autophagy, was significantly increased in licorice-treated cells. We also used TEM to observe the ultrastructure of autophagy in licorice-treated LNCaP cells. Whereas control cells exhibited few autophagic features, numerous autophagic vacuoles and empty vacuoles were observed in LNCaP cells treated with licorice by TEM (**Figure 3C**). We then calculated the area of cytoplasm occupied by autophagic vacuoles in 20 licorice-treated and 20 control cell sections. **Figure 3D** shows that licorice treatment significantly increased the production of autophagic vacuoles. Collectively, these results indicate that licorice induced autophagy in LNCaP cells.

Licorice Induced Autophagy-Related and -Independent Cell Death. We next investigated whether autophagy played roles in licorice-induced cell death in LNCaP cells. Either the pan-caspase inhibitor zVADfmk or BafA1, a potent acidification inhibitor that prevents autophagy at a late stage by inhibiting fusion between autophagosomes and lysosomes, could partially protect the cells from licorice-induced death, as assessed by the cell viability assay (LDH release assay) (**Figures 2C** and **4A**). Moreover, zVADfmk had a greater protective effect than BafA1 against licorice-induced cell death. Consistently, determination of cellular DNA content by flow cytometry also shows that treatment of LNCaP cells with zVADfmk resulted in inhibition of licorice-induced apoptosis, as revealed by a decrease in the percentage of cells at the sub-G1 phase (**Figure 4B**). In marked contrast, treatment of BafA1 further increased the percentage of apoptotic cells in LNCaP cells treated with licorice, indicating that apoptosis was triggered in licorice-treated cells when autophagy was inhibited by BafA1 (*36*). Furthermore, the apoptosis induced by licorice in conjunction with BafA1 could be completely abrogated by zVADfmk treatment. BafA1 could partially protect LNCaP cells from licorice-induced cell death (**Figure 4A**), suggesting that BafA1 may have also protected cells from autophagy-related cell death. To further provide evidence to support this speculation, we performed flow cytometric analysis of acridine orange-stained cells to quantify the AVOs, a characteristic of autophagy. Treatment of BafA1 significantly abrogated the formation of AVOs induced by licorice in LNCaP cells (**Figure 4C**). These results substantiate the observation that licorice induced autophagy in LNCaP cells.

Licorice Affected the Expression of Beclin 1 and Bcl-2. Modulation of beclin 1 and Bcl-2 expression can affect the induction of autophagy (*15*, *37*, *38*). Licorice and its component LA have been

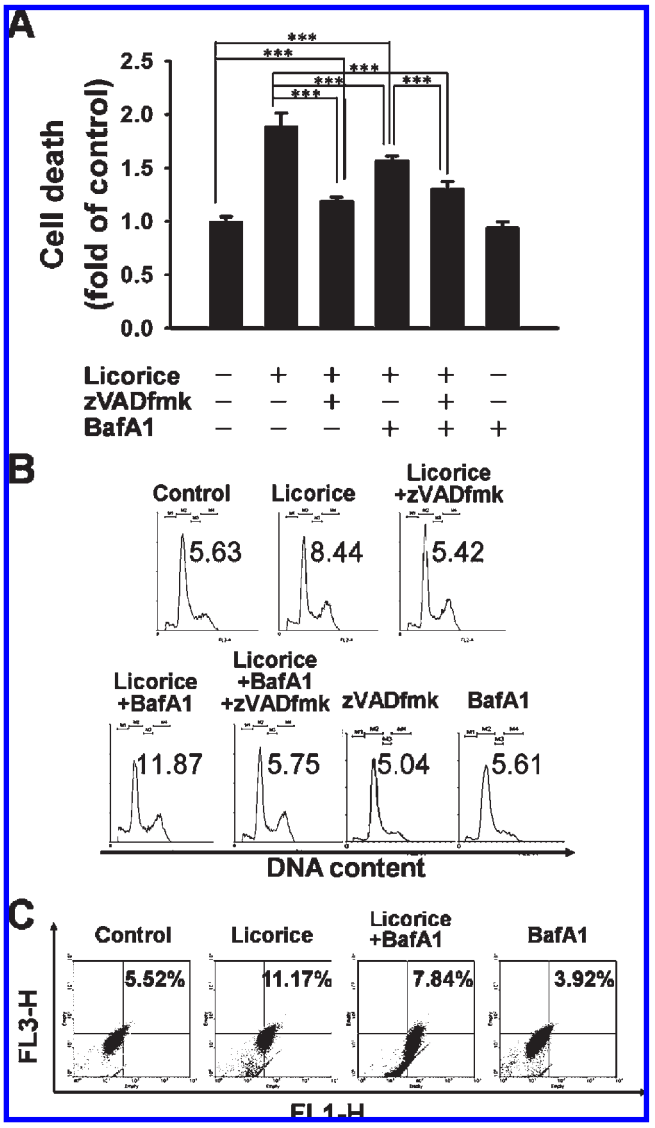

Figure 4. Licorice induced apoptotic and autophagy-related cell death in LNCaP cells. Cells were treated with licorice (228 μg/mL) alone or in the presence of zVADfmk (50 μM) or BafA1 (10 nM) for 24 h. (**A**) Effects of the pan-caspase inhibitor zVADfmk and the autophagy inhibitor BafA1 on licorice-induced cell death. Cell survival was determined by the cell viability assay (LDH release assay). Note that zVADfmk and BafA1 partially protected cells from licorice-induced death. Values are mean ± SD (n = 5). ***, P < 0.001. (**B**) DNA histograms of LNCaP cells after treatment with licorice in the presence or absence of zVADfmk or BafA1. The value shown in each histogram is the percentage of apoptotic cells measured by sub-G1 cell population. (**C**) Quantification of AVOs, which are acidic vesicular organelles characteristic of autophagy, with acridine orange staining using flow cytometry. Cells were stained with acridine orange and analyzed by flow cytometry using FL3-H mode (>650 mm) to value the bright red fluorescence and FL1-H mode (500−550 nm) to value the green fluorescence. Note that treatment of licorice increased formation of AVOs, which could be inhibited by BafA1. The acidic compartments fluoresce bright red, whereas the cytoplasm and nucleolus fluoresce bright green and dim red. The value shown in each histogram is the percentage of cells that fluoresce bright red, indicating AVOs. Similar results were observed in two independent experiments.

shown to induce apoptosis through the cleavage of Bcl-2 and overexpression of Bax in human breast cancer cells (*10*, *39*). In human leukemic cells, Bcl-2 is essential for cell survival, and its down-regulation results in autophagy-related cell death (*40*).

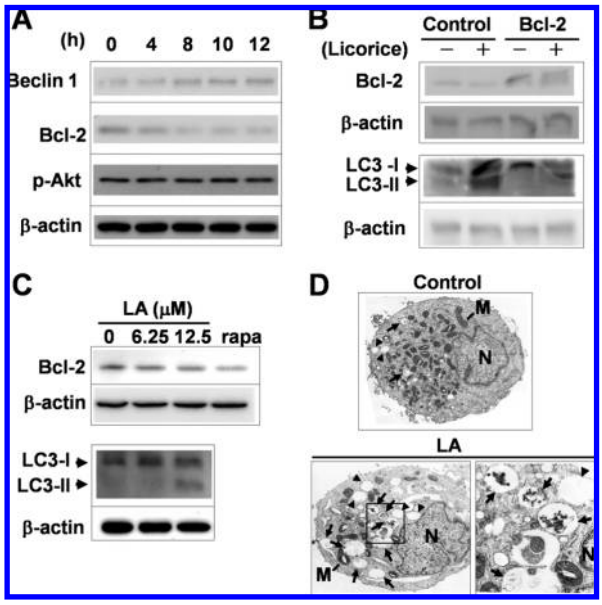

Figure 5. Licorice or LA enhanced beclin1 expression but decreased Bcl-2 expression and induced autophagy in LNCaP cells. (**A**) Treatment with licorice increased beclin1, but decreased Bcl-2 expression. Cells were treated with licorice (228 μg/mL) for different time intervals followed by immunoblot analysis. Note that phosphorylated Akt (p-Akt) expression remained constant during the 12 h treatment period. (**B**) Immunoblot analysis reveals that treatment of licorice (228 μg/mL) for 24 h down-regulated the expression of Bcl-2 with concomitant enhancement of the conversion of LC3-I to LC3-II, which is indicative of autophagic induction, in not only LNCaP cells but also Bcl-2-overexpressing LNCaP cells. (**C**) Treatment with LA for 24 h also down-regulated the expression of Bcl-2 with concomitant enhancement of the conversion of LC3-I to LC3-II in LNCaP cells. Rapamycin (rapa, 5 nM) treatment served as the positive control for down-regulation of Bcl-2. (**D**) Ultrastructure of LNCaP cells treated with LA (12.5 μM) for 24 h was observed by TEM. Cells were fixed and observed for autophagic vacuoles (5000× magnification) by TEM. The lower right panel shows the magnified image (20000× magnification) of the area indicated by the box in the lower left panel. Autophagic vacuoles and empty vacuoles in the cytoplasm of LNCaP cells were denoted by arrows and arrowheads, respectively. N, nucleus. M, mitochondrion.

In addition, a plant lectin from *Polygonatum cyrtonema* also induces apoptosis and autophagy in human melanoma cells by regulation of Bcl-2 and Bcl-2 family proteins (*41*). We next detected the expression of beclin 1 and Bcl-2 in licorice-treated LNCaP cells by immunoblotting. **Figure 5A** shows that treatment of licorice (228 μg/mL) dramatically increased the expression of beclin 1, but decreased the expression of Bcl-2 at 8 h post-treatment. Moreover, the levels of beclin 1 and Bcl-2 remained relatively constant from 8 h onward. We further used Bcl-2-overexpressing LNCaP cells and their control counterparts to investigate the correlation of Bcl-2 level with licorice-induced autophagy. LC3 is a reliable marker of autophagosome formation, and the relative amount of LC3-II reflects the abundance of the autophagosomes. Autophagic cells are characterized biochemically by cleavage of LC3 (*29*). Immunoblot analysis reveals that treatment with licorice in LNCaP cells down-regulated the expression of Bcl-2 with concomitant enhancement of the conversion of LC3-I to LC3-II, which is indicative of autophagic induction (**Figure 5B**). Notably, in Bcl-2-overexpressing cells, licorice treatment also reduced the level of Bcl-2 expression and concomitantly increased the level of LC3-II. Therefore, modulation of Bcl-2 expression affected autophagic formation induced by licorice. The apoptosis and autophagy-related cell death is

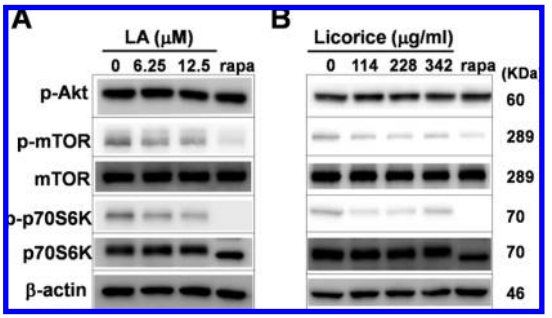

Figure 6. Licorice and LA directly inhibited the mTOR pathway in LNCaP cells. Cells were treated with different concentrations of LA (**A**) or licorice (**B**) for 24 h followed by immunoblotting. Rapamycin (rapa, 5 nM) treatment served as the positive control for inhibition of the phosphorylation of mTOR. Note that the levels of p-mTOR and p-p70S6K were decreased following LA or licorice treatment, whereas the levels of p-Akt, total mTOR, and total p70S6K remained constant. Similar results were observed in at least three independent experiments.

triggered by down-regulation of Bcl-2 (*40*). Licorice induced dissipation of the MMP and thereby resulted in cell death, which may be attributed to the reduced expression of Bcl-2 in licorice-treated cells (*42*). Simultaneously, in order to protect cells from undergoing apoptotic cell death induced by impaired mitochondria, the mechanism of autophagy is executed to remove dysfunctional mitochondria (*12*, *43*). Physiological levels of autophagy are essential for normal cellular homeostasis. In contrast, excessive, nonphysiological levels of autophagy promote autophagy-dependent cell death. The relative amounts of beclin 1 and Bcl-2 complexed with each other within a cell govern the threshold for transition from cell homeostasis to cell death (*15*). Licorice extract may have induced an imbalance between Bcl-2 and beclin 1 levels in LNCaP cells, leading to the promotion of autophagy (*15*, *44*). It was shown that the Akt/mTOR pathway, which is activated in many types of cancer, inhibited autophagy (*12*, *17*). In LNCaP cells, a relatively constant level of activated (phophorylated)-Akt (p-Akt) was detected during 12 h of licorice treatment (**Figure 5A**). These results suggest that modulation of Bcl-2 and beclin 1 expression may have contributed to apoptosis and autophagy formation in licorice-treated LNCaP cells.

LA Also Induced Autophagy. Previous papers have shown that licorice components can modify the expression or phosphorylation of Bcl-2 in cancer cells (*3*, *5*, *7*, *10*). To determine the effect of the licorice component LA on the levels of Bcl-2 and LC3-II, LNCaP cells treated with LA or rapamycin, an autophagy inducer (*45*), were assessed by immunoblotting. **Figure 5C** shows that LA, similar to licorice extract, down-regulated the expression of Bcl-2 with concomitant enhancement of the conversion of LC3-I to LC3-II (**Figure 5C**). Of note, the expression of beclin 1 was not affected in LA-treated LNCaP cells (data not shown). We further applied TEM to confirm the ultrastructure of autophagy in LA-treated LNCaP cells. Consistent with the result of enhanced conversion of LC3-I to LC3-II by LA, there were numerous autophagic vacuoles, empty vacuoles, and fewer mitochondria in LA-treated LNCaP cells, in contrast to the presence of fewer autophagic vacuoles and more mitochondria in the control cells (**Figure 5D**). Collectively, these results indicate that treatment of LA, similar to licorice, induced autophagy in LNCaP cells.

Licorice and LA Directly Inhibited the mTOR Pathway. Autophagy formation is associated with various signaling pathways. Rapamycin, an mTOR inhibitor, exerts its antitumor effect by

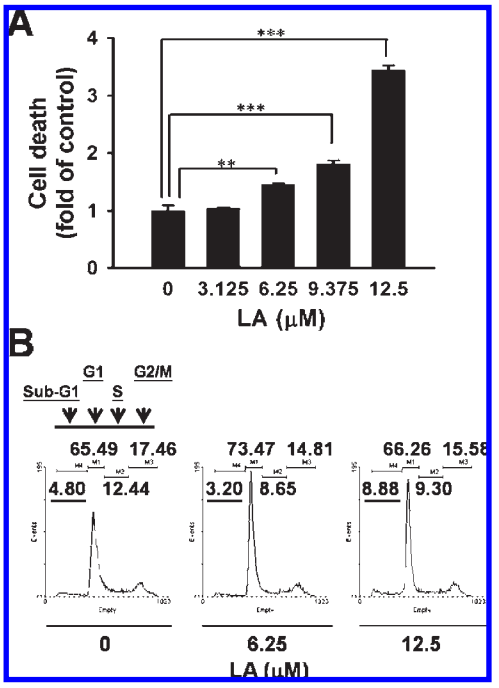

Figure 7. LA induced cell death in LNCaP cells. (**A**) Cell death in LNCaP cells treated with various concentration of LA for 24 h was determined by the cell viability assay (LDH release assay). Cell death is presented as the ratio of the LDH released from the LA-treated cells to that from the untreated control cells. Data are mean ± SD ($n = 3$). Similar results were observed in three independent experiments. ***, $P < 0.001$; **, $P < 0.01$. (**B**) Representative flow histograms depicted subdiploid fraction in LNCaP cells treated with LA for 24 h. The values shown in each histogram are the percentages of cells in different cell cycle phases. The percentage of sub-G1 cell population is underlined. Note that the subdiploid fraction was observed only in cells treated with 12.5 μM LA.

inducing autophagy, suggesting that in malignant cancer cells it disrupts the PI3K/Akt signaling pathway (*46*). Ceramide was shown to trigger autophagy by interfering with the mTOR signaling pathway and by alleviating the inhibitory effect of Bcl-2 on autophagy (*47*, *48*). Apart from beclin 1 and Bcl-2, the Akt/mTOR/p70S6K signaling pathway also negatively regulates autophagy (*17*, *44*). As LA modified Bcl-2 but not beclin 1 expression, we next examined the roles of the Akt/mTOR/p70S6K signaling pathway in licorice- or LA-induced autophagy. In LNCaP cells treated with LA, the levels of p-Akt remained unchanged (**Figure 6A**). Nevertheless, treatment of LA decreased the phosphorylation of mTOR and its downstream target p70S6K, indicating down-regulation of the mTOR/p70S6K pathway by LA. However, total mTOR and p70S6K levels were not affected by LA treatment. Furthermore, very similar results were obtained when LA was replaced with licorice extract (**Figure 6B**). The effects of LA or licorice extract on the activation of Akt, mTOR, and p70S6K pathways in LNCaP cells were similar to those induced by rapamycin (*44*). In addition, Bcl-2 expression levels predict the cellular effects of mTOR inhibitors in human ovarian carcinoma. These results indicate that down-regulation of Bcl-2 combined with rapamycin may be useful for treating ovarian cancer (*49*). Taken together, these results suggest that induction of autophagy by either licorice extract or LA in LNCaP cells was associated with direct inhibition of the mTOR activity, but not through the suppression of Akt phosphorylation. Although autophagy is enhanced by imbalanced expressions of beclin 1 and Bcl-2 (*15*, *44*) or suppression of the mTOR signal cascade (*44*), excess autophagic vacuoles that mediate elimination

of essential cell organelles still eventually led to autophagy-related cell death (*12*, *15*). We presumed that licorice extract or LA may induce excessive autophagy formation by down-regulation of Bcl-2 and inhibition of mTOR activity.

LA Induced Cell Death in a Dose-Dependent Manner. Previous papers indicated that LA induces cell cycle arrest and apoptosis in breast and prostate cancer cells (*1*, *10*). We used the cell viability assay (LDH release assay) to assess cell death in LNCaP cells following LA treatment for 24 h. Significant cell death was observed when 6.25 μM LA was applied to LNCaP cells (**Figure 7A**). Analysis of cellular DNA content reveals that sub-G1 population (apoptotic cells) did not increase, whereas accumulation of cells in the G1 phase of the cell cycle could be observed in the cells treated with 6.25 μM LA (**Figure 7B**). Furthermore, in cells treated with 12.5 μM LA, the extent of cell death (**Figure 7A**) was more evident than the degree of apoptosis (**Figure 7B**), suggesting that apoptosis may not be the sole mechanism contributing to LA-induced cell death. Because licorice extract induced apoptotic and autophagy-related cell death, and LA treatment also resulted in apoptosis, autophagy, and cell death, it is tempting to speculate that LA, a component of licorice, may also induce autophagy-related cell death. The mechanism underlying the cell death induced by LA is currently being investigated.

In conclusion, our findings reported here not only add a novel function for licorice and LA to induce autophagy but also suggest a potential strategy for the use of licorice to induce caspase-dependent and autophagy-related cell death in prostate cancer cells.

ABBREVIATIONS USED

AVOs, acidic vesicular organelles; BafA1, bafilomycin A1; GFP, green fluorescent protein; LA, licochalcone-A; LC3, microtubule-associated protein 1 light chain 3; LDH, lactate dehydrogenase; MBB, monobromobimane; MDC, monodansylcadaverine; MMP, mitochondrial membrane potential; mTOR, mammalian target of rapamycin; p70S6K, ribosomal S6 kinase; zVADfmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

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