Licochalcone A induces T24 bladder cancer cell apoptosis by increasing intracellular calcium levels

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Abstract. Licochalcone A (LCA) has been reported to significantly inhibit cell proliferation, increase reactive oxygen species (ROS) levels, and induce apoptosis of T24 human bladder cancer cells via mitochondria and endoplasmic reticulum (ER) stress-triggered signaling pathways. Based on these findings, the present study aimed to investigate the mechanisms by which LCA induces apoptosis of T24 cells. Cultured T24 cells were treated with LCA, and cell viability was measured using the sulforhodamine B assay. Apoptosis was detected by flow cytometry with Annexin V/propidium iodide staining, and by fluorescent microscopy with Hoechst 33258 staining. The levels of intracellular free calcium ions were determined using Fluo-3 AM dye marker. Intracellular ROS levels were assessed using the 2',7'-dichlorodihydrofluorescein diacetate probe assay. The mitochondrial membrane potential was measured using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazole carbocyanine iodide. Furthermore, the mRNA expression levels of B-cell lymphoma (Bcl)-extra large, Bcl-2-associated X protein, Bcl-2-interacting mediator of cell death, apoptotic protease activating factor-1 (Apaf-1), calpain 2, cysteinyl aspartate specific proteinase (caspase)-3, caspase-4 and caspase-9 were determined using reverse transcription semiquantitative and quantitative polymerase chain reaction analyses. Treatment with LCA inhibited proliferation and induced apoptosis of T24 cells, and increased intracellular Ca^{2+} levels and ROS production. Furthermore, LCA induced mitochondrial dysfunction, decreased mitochondrial membrane potential, and increased the mRNA expression levels of Apaf-1, caspase-9 and caspase-3. Exposure of T24 cells to LCA also triggered calpain 2 and caspase-4 activation, resulting in apoptosis. These findings indicated that LCA increased intracellular Ca^{2+} levels, which may be associated with mitochondrial dysfunction. In addition, the ER stress pathway may be considered an important mechanism by which LCA induces apoptosis of T24 bladder cancer cells.

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Introduction

Natural herbal medicines have long been used to treat cancer. Biomedical research and cancer treatment clinical trials have provided evidence regarding the use of herbal medicines; therefore, they are increasingly being accepted as a complementary and alternative treatment (1). In addition, natural medicines have been reported to have an important role in human health, particularly certain well-studied plants, including *Taxus chinensis* (*Taxus madia*) (2), Radix *Sophorae flavescentis* (*Sophora flavescens*) (3), *Alkanna tinctoria, Lithospermum erythrorhizon* and licorice (*Glycyrrhiza* L) (4). Licorice is one of the most commonly prescribed herbs in Chinese Traditional Medicine, and has been used for >2,000 years. The effects of licorice have been reported on various diseases, ranging from microbial infection to cancer (5-7). However, since herbs usually contain numerous chemical compositions, the mechanism of action of these herbs is currently unclear. Recently, several chemical ingredients have been isolated and proved to contribute to the activities of licorice. The main bioactive constituents of licorice include triterpene saponins and various types of flavonoids (8).

Licochalcone A (LCA) is a chalcone compound isolated from licorice root (Radix *Glycyrrhizae*), which is known for its numerous biological activities, including anti-inflammatory (9) antimicrobial (10), antioxidant (11) and anticancer effects (12).

Apoptosis, or 'programmed cell death' (PCD), is a normal event that occurs in multicellular organisms (13). Previous studies have suggested that reactive oxygen species (ROS) have an important role in the mitochondrial apoptotic pathway (14), and have been shown to be involved in endoplasmic reticulum (ER) stress-induced apoptosis (15,16). In addition, increases in intracellular Ca^{2+} levels may contribute to ROS accumulation and ER stress (17).

Ca^{2+}, which is one of the most versatile second messengers, regulates various cellular functions, including contraction, secretion, metabolism, gene expression, cell survival and PCD (18). Calpains are Ca^{2+}-activated non-lysosomal cysteine

proteases, which have been reported to promote cysteinyl aspartate specific proteinase (caspase)-4 activation during ER stress-induced apoptosis (19-21). Furthermore, it has been reported that LCA induces apoptosis in HepG2 hepatocellular carcinoma cells through induction of ER stress via a phospholipase C gamma 1-, Ca^{2+}- and ROS-dependent pathway (22).

Our previous studies have demonstrated that LCA may significantly increase ROS levels and induce apoptosis of T24 human bladder cancer cells (23). However, the underlying molecular mechanism remains unclear. The present study aimed to further elucidate the mechanism by which LCA induces apoptosis of T24 cells.

Materials and methods

Reagents. LCA (purity ≥98%) was purchased from Zhongxin Pharmaceutical Group Corp., Ltd. (Tianjin, China). Roswell Park Memorial Institute (RPMI) 1640 medium was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Super neonatal bovine serum (NBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Annexin V/propidium iodide (PI) apoptosis kit was purchased from Nanjing KeyGen Biotech. Co. Ltd. (Nanjing, China). Molecular Probes 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Thermo Fisher Scientific, Inc.,), ethylene glycol tetraacetic acid (EGTA), Fluo-3 AM ester, and Hoechst 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). BAPTA-AM was purchased from AAT Bioquest, Inc. (Sunnyvale, CA, USA).

Cell culture and treatment. The T24 human bladder cancer cells were purchased from the Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI 1640 medium supplemented with 10% NBS (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were allowed to attach for 24 h prior to treatment. LCA was dissolved in dimethyl sulfoxide (DMSO) and diluted with fresh medium to achieve the desired concentration. The final concentration of DMSO did not exceed 0.2% in the fresh medium, and DMSO at this concentration is known to have no significant effect on cell viability.

Cell viability assay. Cell viability was measured using the sulforhodamine B (SRB) assay (24). Briefly, T24 cells were trypsinized and seeded into 96-well plates at $1.0x10^5$ cells/ml. Subsequently, the cells were exposed to LCA (0, 20, 40 and 80 μM) for 24 h, followed by a further 24 h incubation in fresh medium. The T24 cells were then fixed with trichloroacetic acid and stained for 30 min with 0.4% (wt/vol) SRB (Sigma-Aldrich) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 150 ml DMSO for determination of optical density was detected at a wavelength of 490 nm using a Varioskan Flash 3001 plate reader (Thermo Fisher Scientific, Inc.).

Quantification of apoptosis by flow cytometry. Apoptosis was assessed using Annexin V-fluorescein isothiocyanate (FITC) and PI labeling, as previously described (25). Cells were treated with 0, 20, 40 and 80 μM LCA for 24 h. Subsequently, the cells were washed twice with phosphate-buffered saline (PBS), and were resuspended in staining buffer containing 5 μl (1 mg/ml) PI and 5 μl Annexin V-FITC. Double-labeling was performed at room temperature for 10 min in the dark prior to flow cytometric analysis. Cell staining was detected using a FACStar flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Determination of morphological alterations. Alterations in the nuclear morphology of apoptotic cells were observed by labeling the cells with the nuclear stain Hoechst 33258, and examining them under a fluorescent microscope. After treatment with 0, 20, 40 and 80 μM LCA for 24 h, the cells were fixed in formaldehyde (40 g/l) in PBS for 20 min, followed by Hoechst 33258 (10 mg/l) staining for 30 min in the dark at 37˚C. Nuclear morphology was subsequently observed under a fluorescence microscope (MIC00266; Zeiss, Oberkochen, Germany) (26).

Detection of Ca^{2+} concentration. Ca^{2+} concentration was measured by Fluo-3 AM staining and microscopy. Briefly, T24 cells were incubated with or without Ca^{2+} chelators (200 μM EGTA and 10 μM BAPTA-AM) for 1 h prior to 5 μM LCA treatment for 24 h. Subsequently, the cells were harvested and washed twice, and were resuspended in Fluo 3 AM (5 μM) at 37˚C for 30 min. After washing three times, the stained cells were observed under a computer-assisted microscope (MIC00266; Carl Zeiss AG, Oberkochen, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Detection of intracellular ROS levels. The intracellular levels of ROS in T24 cells were assessed using H_2DCFDA (27). Briefly, cells were incubated with 55 μM LCA or with 10 μM BAPTA-AM (a chelator of cytosolic Ca^{2+}) for 1 h prior to LCA treatment. The treated cells were then washed in PBS and incubated with 30 μM H_2DCFDA for 30 min at 37˚C. Fluorescence was detected using a fluorescent plate reader at 485/525 nm excitation/emission wavelengths (Varioskan Flash 3001; Thermo Fisher Scientific, Inc.) and data were expressed as median fluorescence intensity. The stained cells were then observed under a computer-assisted microscope (MIC00266; Carl Zeiss AG).

Measurement of mitochondrial membrane potential (MMP). The MMP was assessed using a dual-emission potential-sensitive probe, 5,5',6, 6'-tetra-chloro-1, 1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1; KeyGen Biotech Co., Ltd., Nanjing, China). The ratio of red to green fluorescence of JC-1 depends solely on membrane potential, with a decrease being indicative of membrane depolarization (28). Briefly, the cells were exposed to LCA (0, 20, 40 or 80 μM) for 4, 8, 16 or 24 h. Subsequently, the cells were loaded with 2 mg/l JC-1 at 37˚C for 20 min, and were analyzed using a plate reader (Varioskan Flash 3001; Thermo Fisher Scientific, Inc.).

Reverse transcription semiquantitative and quantitative polymerase chain reaction (PCR). Total RNA was extracted from

Table I. Polymerase chain reaction primer sequences.

Primer	Forward	Reverse
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG
Bcl-xL	GCATATCAGAGCTTTGAACAGGT	TAGGTGGTCATTCAGGTAAGTGG
Bax	ACGAACTGGACAGTAACATGGAG	CAGTTTGCTGGCAAAGTAGAAAAG
Bim	CACATGAGCACATTTCCCTCT	AAGGCACAAAACCTGCAGTAA
Caspase-3	CTGGACTGTGGCATTGAGAC	ACAAAGCGACTGGATGAACC
Caspase-4	TGAACTGGAAGGAAGAGGAA	GCGGTTGTTGAATATCTGGA
Caspase-9	CAGTGGGCTCACTCTGAAGACC	ACGCGTTACTGGCATTGAGG
Apaf-1	TGGAATGGCAGGCTGTGGGA	TGCACTCCCCCTGGGAAACA
Calpain 2	GCAGCCATTGCCTCCCTCAC	ACCTCCACCCACTCGCCGTA

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Bcl-xL, B-cell lymphoma (Bcl)-extra large; Bax, Bcl-2-associated X protein; Bim, Bcl-2-interacting mediator of cell death; Apaf-1, apoptotic protease activating factor-1.

T24 cells using TRIzol (Sangon Biotech Co., Ltd., Shanghai, China). RNA quality was determined using the A260/A280 ratio and 1.5% agarose gel electrophoresis. cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase with a First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). The synthesized cDNA was amplified by Riboloek Nase-free ddH_2O (8 μl), template RNA (3 μl), Oligo(dT)18 (1 μl) to a final volume of 12 μl. Tubes were placed into the C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 70˚C for 5 min, following which 5X reaction buffer (4 μl), Riboloek Nase Indihitor (1 μl), dNTP M-M RT RTase (1 μl) and RNase DEPC-treated water to a final volume of 20 μl were added and incubated at 42˚C 1 h for denaturation and 70˚C 5 min for annealing. The synthesized cDNA was amplified by Oligo$(dT)_{18}$, according to the instructions of a PCR Amplification kit (Fermentas; Thermo Fisher Scientific, Inc.). The PCR primers (synthesized by Sangon Biotech Co., Ltd.; presented in Table I) and their cycling conditions were set as indicated. The PCR reaction volume consisted of 12.5 μl 2X PCR Master (Sangon Biotech Co., Ltd.), 3 μl cDNA template and 0.5 μl of each primer. The cycling conditions were as follows: Pre-denaturation at 94˚C for 3 min, with 30-35 cycles of denaturation at 94˚C for 30 sec, annealing for 30 sec, extension at 72˚C and a final extension at 72˚C for 10 min, using a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc.). The gene products were quantified using agarose gel electrophoresis (Biodee Biotechnology Co., Ltd., Beijing, China) and a Bio-Rad gel imaging system (Bio-Rad Laboratories, Inc.).

The quantitative PCR analysis was carried out according to the manufacturer's protocol of the Taqman One-Step PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Total cDNA (2 μl) was added per 25 μl reaction, alongside 0.5 μl sequence-specific primers and 12.5 μl SYBR Premix Ex Taq. All target gene primers and probes were purchased commercially (Sangon Biotech Co., Ltd.). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The cycling conditions were as follows: Pre-denaturation at 95˚C for 4 min, denaturation at 95˚C for 5 sec, annealing for 30 sec, extension at 72˚C and a final extension at 72˚C for 10 min, using a Rotor-Gene Q Real time PCR machine (Qiagen China Co., Ltd, Shanghai, China). Relative expression levels of the target genes were calculated based on the $2^{-\Delta\Delta Cq}$ method of relative quantification (29), according to the following equation: Relative expression level = $2^{(Cq\ value\ of\ GAPDH\ -\ Cq\ value\ gene\ of\ interest)}$. The primer sequences are presented in Table I.

Statistical analysis. Data are presented as the mean ± standard from at least three independent experiments. Data were evaluated by one-way analysis of variance followed by Student-Newman-Keuls test, uding Origin software, version 8.0 (OriginLab, Northampton, MA, USA). In all cases, $P<0.05$ was considered to indicate a statistically significant difference.

Results

LCA induces cell apoptosis in T24 human bladder cancer cells. To examine cell viability *in vitro*, the SRB assay was used to determine the inhibitory effects of LCA on proliferation. A total of 24 h post-LCA treatment, LCA reduced the proliferation of T24 cells in a dose-dependent manner; the half maximal inhibitory concentration was ~55 μM ($P=4.3x10^{-13}$; Fig. 1A). Subsequently, it was investigated whether LCA was able to induce cell death through an apoptotic mechanism. Annexin V-FITC and PI double-labeling was used for the detection of phosphatidylserine externalization, a hallmark of early phase apoptosis. Compared with the control group, a high proportion of Annexin V^+ labeling was detected in cells treated with LCA, thus indicating that they were in the early phase of apoptosis ($P=1.3x10^{-5}$; Fig. 1B and C). These results indicate that LCA significantly induced apoptosis in T24 cells.

LCA induces alterations in nuclear morphology. Typical apoptotic morphological alterations, as indicated by condensed nuclei and nuclear fragmentation, were apparent after exposure to 40 μM LCA. Apoptotic nuclear alterations were markedly increased in the cells pretreated with 80 μM LCA (Fig. 2).

LCA induces Ca^{2+} release. Following treatment of T24 cells with LCA, Ca^{2+} levels were significantly increased compared

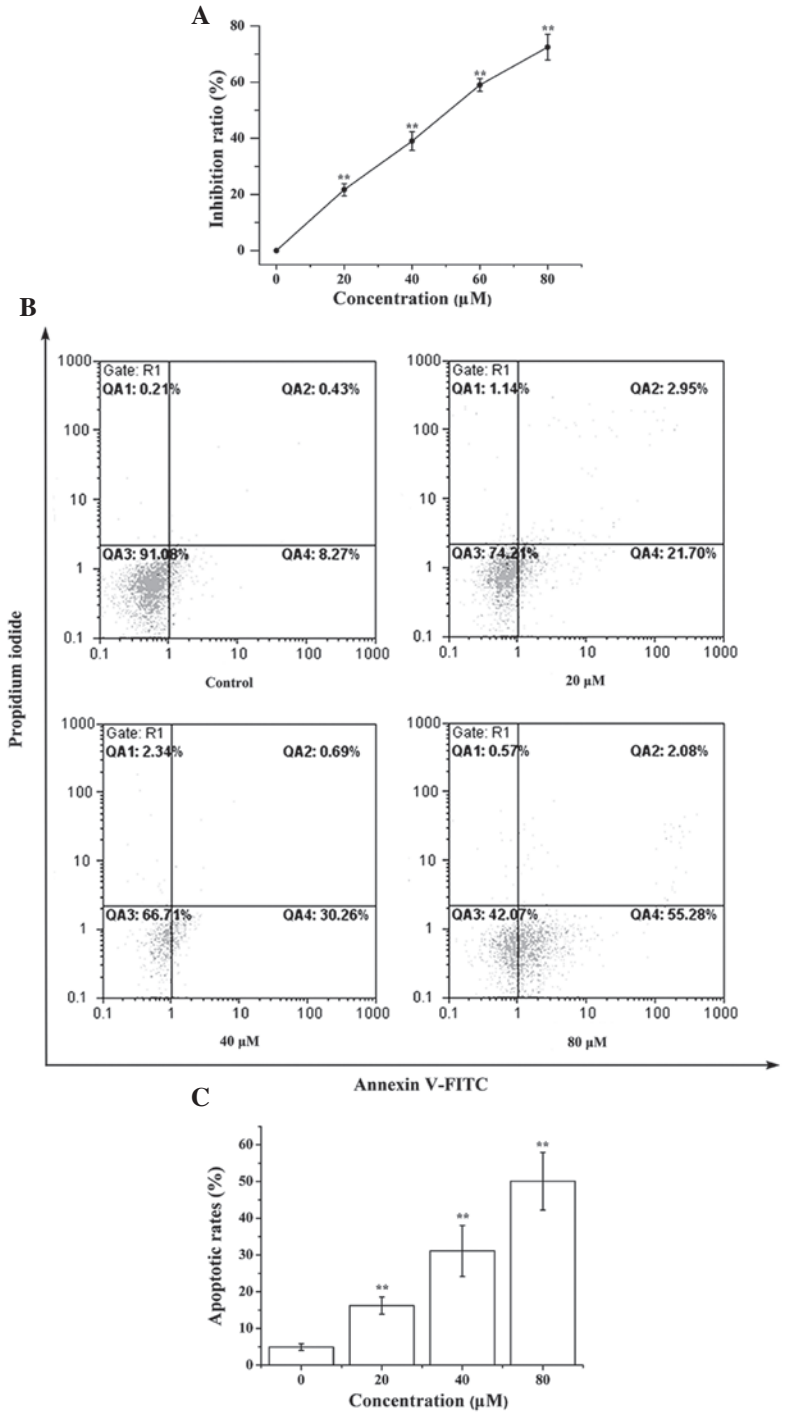

Figure 1. Licochalcone A (LCA) induced the apoptosis of T24 cells. (A) Effects of LCA on T24 cell proliferation and survival. The inhibitory ratio of LCA on cell proliferation was determined using the sulforhodamine B assay 24 h post-treatment (0, 20, 40, 60 and 80 μM LCA). (B and C) Cells were treated with 0, 20, 40 and 80 μM LCA for 24 h, and the percentage of apoptotic cells was analyzed by flow cytometric analysis of Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide double staining (n=3). Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 compared with the untreated control group.

with the control group. These results indicate that LCA promoted Ca^{2+} release in a time-dependent manner (30 min, $P=4.3x10^{-13}$; 2 h, $P=1.2x10^{-5}$; 4 h, P=0.0084; Fig. 3A). In order to investigate whether LCA could induce an increase in cytosolic Ca^{2+} through extracellular or intracellular Ca^{2+} pools, the T24 cells were pretreated with EGTA (an extracellular Ca^{2+} chelator) and BAPTA-AM (an intracellular Ca^{2+} chelator). EGTA and BAPTA-AM significantly suppressed LCA-induced Ca^{2+} release ($P=1.9x10^{-6}$ and $P=2.2x10^{-6}$, respectively; Fig. 3B). Furthermore, BAPTA-AM could attenuate LCA-induced apoptosis, whereas EGTA had no inhibitory effects on apoptosis (Fig. 3C). Collectively, these results indicate that LCA mainly induced release of intracellular Ca^{2+} to promote apoptosis.

To determine whether cytosolic Ca^{2+} release is a signal leading to ROS accumulation in LCA-induced apoptosis,

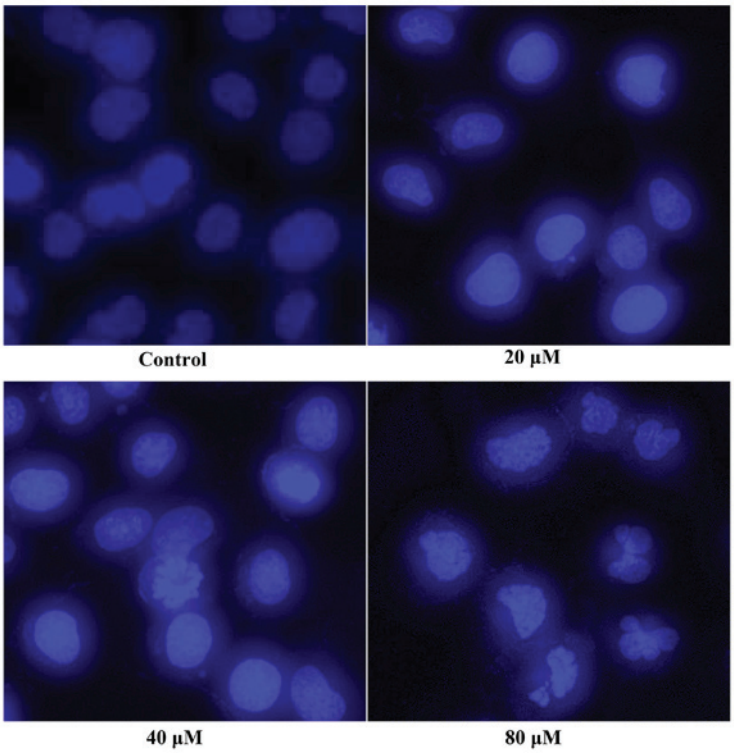

Figure 2. Morphologic alterations in T24 cells. Cells were incubated with 0, 20, 40 and 80 μM licochalcone A for 24 h and stained with the DNA-binding fluorochrome Hoechst 3258 (magnification, x400).

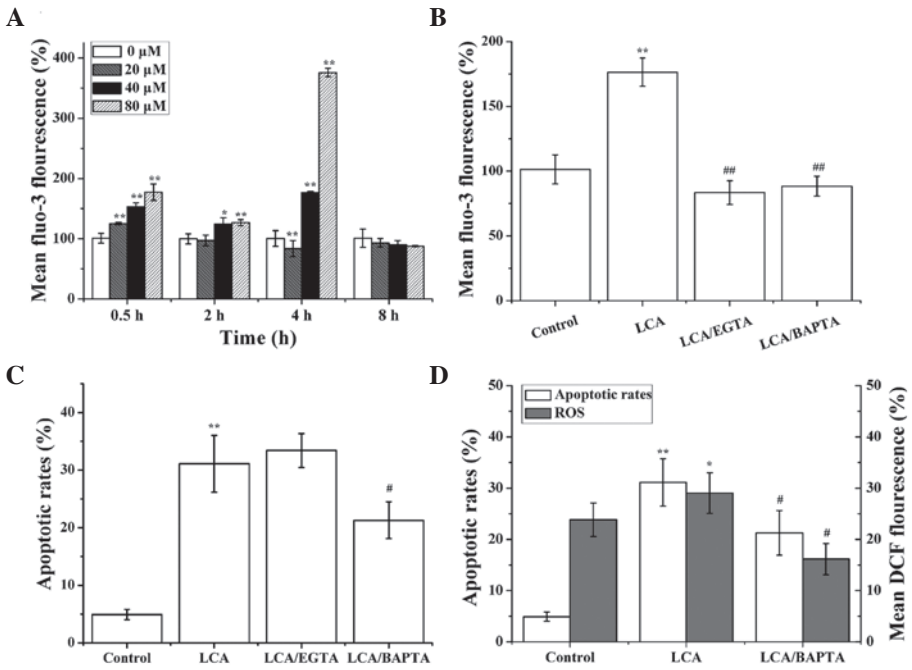

Figure 3. Licochalcone A (LCA) induced Ca^{2+} release and mitochondrial dysfunction in T24 cells. (A) Intracellular calcium fluorescent intensity following treatment with various concentrations of LCA. T24 cells were incubated with 0, 20, 40 and 80 μM LCA for 0.5, 2, 4 and 8 h. Effects of ethylene glycol tetraacetic acid (EGTA and BAPTA-AM on (B) intracelluar Ca^{2+} levels and (C) apoptotic rate; T24 cells were pretreated for 24 h with LCA (55 μM) followed by stimulation with EGTA (200 μM) or BAPTA-AM (10 μM). (D) Effects of BAPTA-AM on apoptotic rates and reactive oxygen species (ROS); T24 cells were pretreated for 24 h with LCA (55 μM) followed by stimulation with BAPTA-AM (10 μM). Data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, **P<0.01 compared with the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the LCA-treated group.

T24 cells were pretreated with BAPTA-AM, then treated with LCA for 1 h, or the cells were treated with LCA alone. The levels of ROS generation were lower in the LCA and BAPTA-AM treated group, as compared with in the LCA-treated group (apoptotic rates, P=0.0241; ROS, P=0.0128; Fig. 3D). These results suggest that BAPTA-AM

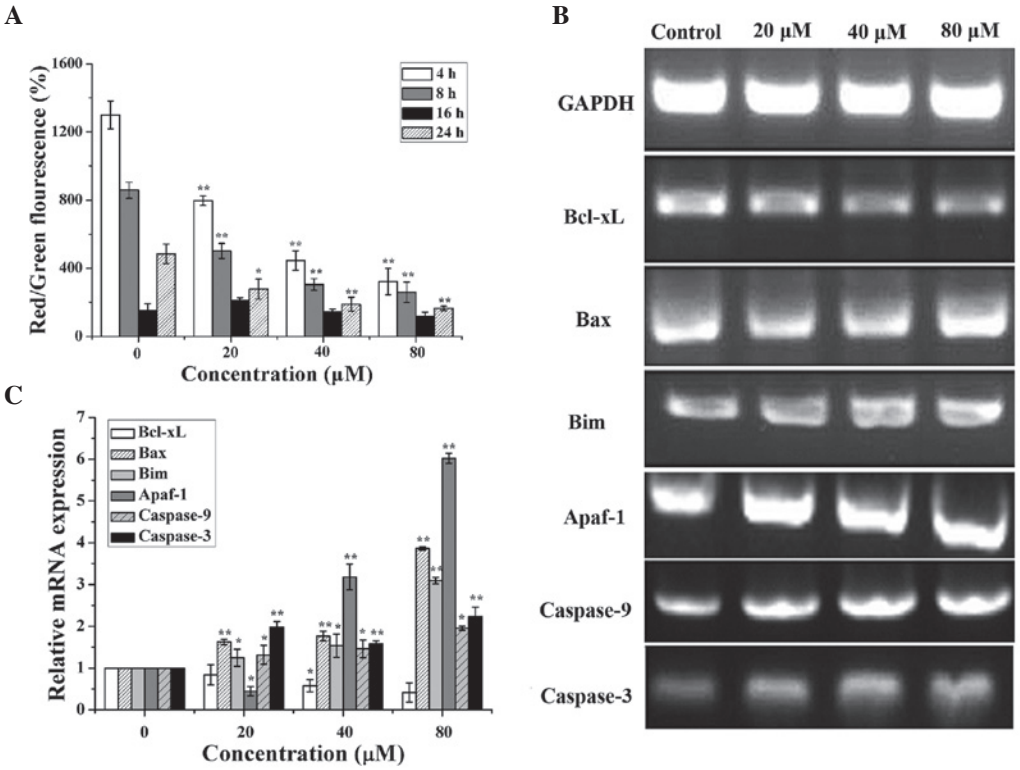

Figure 4. Licochalcone A (LCA) induced mitochondrial dysfunction in T24 cells. (A) T24 cells were incubated with 0, 20, 40 and 80 μM LCA for 4, 8, 16 and 24 h, and the mitochondrial membrane potential was determined. The number of cells with normal polarized mitochondrial membranes (red) compared with the number of cells with depolarized mitochondrial membranes (green) is expressed as a percentage of the total cell number. (B) B-cell lymphoma (Bcl)-extra large (Bcl-xL), Bcl-2-associated X protein (Bax), Bcl-2-interacting mediator of cell death (Bim), apoptotic protease activating factor-1 (Apaf-1), caspase-9 and caspase-3 expression levels were detected by semiquantitative polymerase chain reaction (PCR) following treatment with 0, 20, 40 and 80 μM LCA for 24 h. (C) Changes in the mRNA expression levels of Bcl-xL, Bax, Bim, Apaf-1, caspase-9 and caspase-3 were examined by quantitative PCR analysis. T24 cells were incubated with 0, 20, 40 and 80 μM LCA for 24 h. Data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, **P<0.01 compared with the control group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

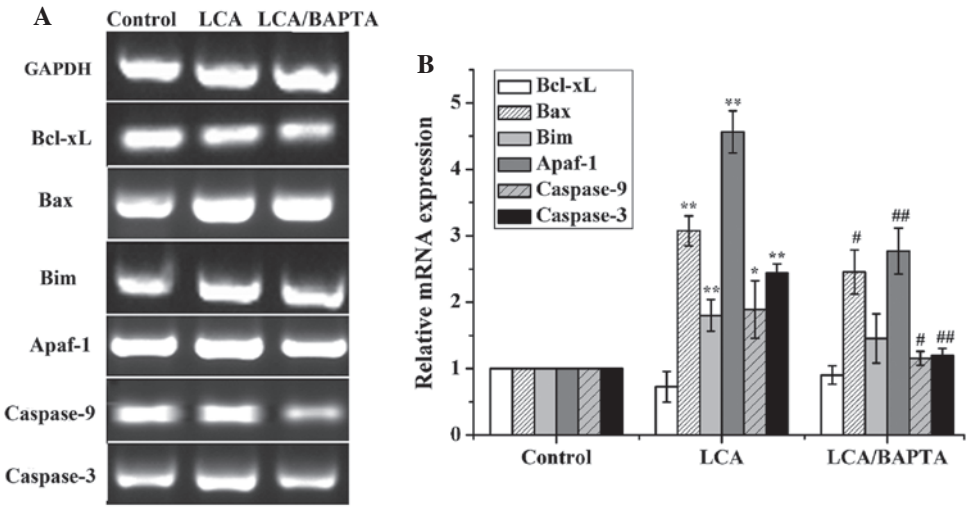

Figure 5. BAPTA-AM mediated the expression of mitochondrial apoptosis-associated genes. (A) B-cell lymphoma (Bcl)-extra large (Bcl-xL), Bcl-2-associated X protein (Bax), Bcl-2-interacting mediator of cell death (Bim), apoptotic protease activating factor-1 (Apaf-1), caspase-9 and caspase-3 expression levels were detected by semiquantitative polymerase chain reaction (PCR). (B) Changes in the mRNA expression levels of Bcl-xL, Bax, Bim, Apaf-1, caspase-9 and caspase-3 were examined by quantitative PCR analysis. T24 cells were pretreated for 24 h with licochalone A (LCA; 55 μM) followed by stimulation with BAPTA-AM (10 μM). Data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, **P<0.01 compared with the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the LCA-treated group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

may decrease LCA-induced apoptosis and ROS generation, thus indicating that cytosolic Ca^{2+} release may act upstream of ROS generation in LCA-treated T24 cells.

Intracellular Ca^{2+} has a critical role in LCA-induced apoptosis of T24 cells. As shown in Fig. 4A, treatment of T24 cells with LCA for 4, 8, 16 and 24 h induced a time-dependent reduc-

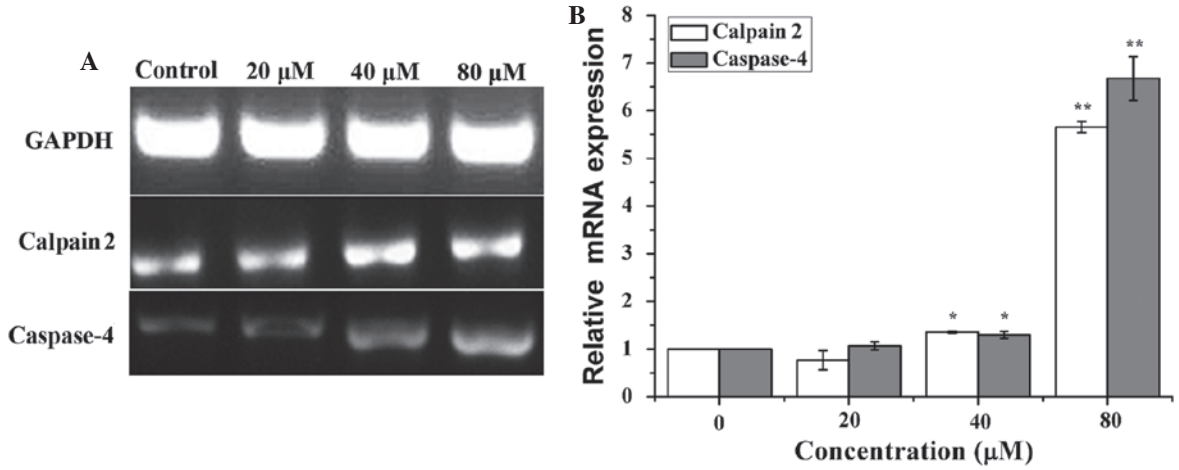

Figure 6. Licochalcone A (LCA) induced endoplasmic reticulum stress in T24 cells. (A) Calpain 2 and caspase-4 expression levels were detected by semiquantitative polymerase chain reaction (PCR). (B) Changes in the mRNA expression levels of calpain2 and caspase-4 were examined by quantitative PCR analysis. T24 cells were incubated with 0, 20, 40 and 80 μM LCA for 24 h. Data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, **P<0.01 compared with the control group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

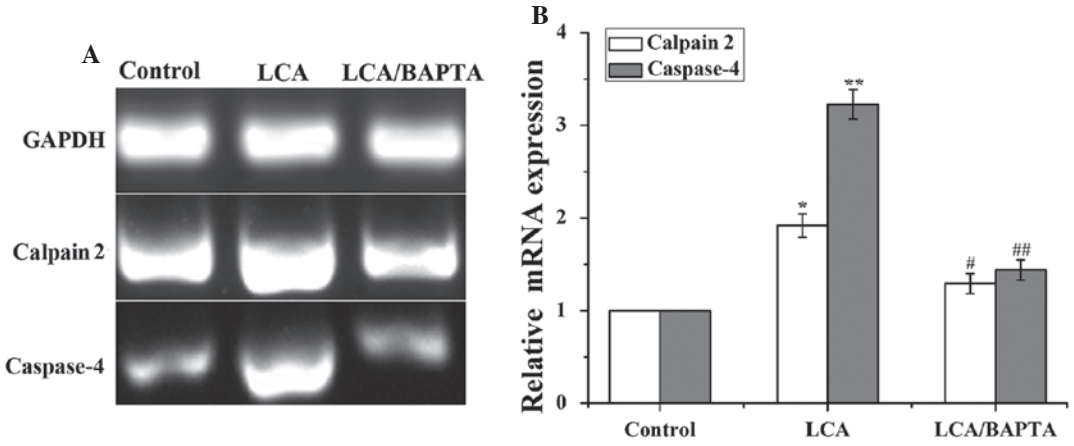

Figure 7. BAPTA-AM mediated the expression of genes involved with endoplasmic reticulum stress-associated apoptosis. (A) Calpain 2 and caspase-4 expression levels were detected by semiquantitative polymerase chain reaction (PCR). (B) Changes in the mRNA expression levels of calpain2 and caspase-4 were examined by quantitative PCR analysis. T24 cells were pretreated for 24 h with licochalcone A (LCA; 55 μM) followed by stimulation with BAPTA-AM (10 μM). Data are presented as the mean ± standard deviation of three separate experiments. *P<0.05, **P<0.01 compared with the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the LCA-treated group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

tion in MMP (P=6.0x10^{-8}, P=9.5x10^{-7}, P=0.0793, P=1.1x10^{-5}, respectively), thus suggesting that LCA-induced cell apoptosis may be associated with mitochondrial dysfunction.

To further explore whether LCA induces apoptosis via the regulation of mitochondrial apoptosis-associated genes, B-cell lymphoma (Bcl)-2-associated X protein (Bax), Bcl-2-interacting mediator of cell death (Bim), Bcl-extra large (xL), apoptotic protease activating factor-1 (Apaf-1), caspase-9 and caspase-3 were detected in T24 cells. The cells were treated with various concentrations of LCA for 24 h. As shown in Fig. 4B and C, the mRNA expression levels of Bcl-xL were downregulated (P=0.052), whereas Bax (P=4.1x10^{-12}), Bim (P=2.4x10^{-8}), Apaf-1 (P=1.8x10^{-14}), caspase-9 (P=0.0107) and caspase-3 (P=0.0001) expression levels were upregulated in a concentration-dependent manner.

To determine whether intracellular Ca^{2+} levels exert a critical role on LCA-induced mitochondrial apoptosis, BAPTA-AM, an intracellular Ca^{2+} chelator, was used. T24 cells were treated with or without BAPTA-AM for 1 h prior to LCA treatment for 24 h. As shown in Fig. 5, compared with the LCA-treated group, Bax (P=0.0374), Apaf-1 (P=5.7x10^{-5}), caspase-9 (P=0.0211) and caspase-3 (P=0.0095) expression were markedly downregulated. However, there was no significant difference in Bcl-xL mRNA expression between the LCA-treated and LCA + BAPTA-AM-treated groups. These data suggest that intracellular Ca^{2+} has a critical role in mitochondrial apoptosis.

LCA induces apoptosis via the ER stress pathway in T24 cells. The involvement of ER stress signaling in the responses triggered by LCA-induced apoptosis was evaluated based on the expression of calpain 2 and caspase-4. As shown in Fig. 6, cells were treated with LCA for 24 h, and calpain 2 and caspase-4 expression levels were increased in a concentration-dependent manner. Subsequently, the T24 cells were treated with or

without BAPTA-AM for 1 h prior to LCA treatment for 24 h,. As shown in Fig. 7, compared with the LCA-treated group, calpain 2 ($P=0.0418$) and caspase-4 ($P=3.8x10^{-5}$) expression levels were downregulated in response to BAPTA-AM treatment. These results suggest that Ca^{2+} is involved in the ER stress-related apoptotic pathway.

Discussion

LCA has been reported to inhibit proliferation and induce apoptosis in various cancer cells, including MCF-7 human breast cancer cells (30) and colon cancer cells (22). We previously reported that LCA induced an increase in cytoplasmic ROS levels, by sensing inner mitochondrial ROS production, and activated caspase-3/caspase-9-mediated mitochondrial apoptotic signaling pathways (31). The present study demonstrated that: i) LCA induced Ca^{2+} release in T24 human bladder cancer cells; ii) LCA predominantly induced an increase in intracellular Ca^{2+} release to promote apoptosis; iii) intracellular Ca^{2+} may cause upstream ROS accumulation in LCA-treated T24 cells; iv) increased intracellular Ca^{2+} levels are involved in LCA-induced T24 cell apoptosis via mitochondrial dysfunction and the ER stress-related pathway. The mechanism by which LCA induces apoptosis may be mediated through increased levels of intracellular Ca^{2+}. Notably, LCA enhanced intracellular Ca^{2+}, induced mitochondrial dysfunction, and activated the apoptotic cascade and ER stress in T24 cells. These findings indicated that intracellular Ca^{2+} may have a prominent role in LCA-induced T24 cell apoptosis via he mitochondria-dependent and ER stress-activated apoptotic signals.

It has been indicated that increased Ca^{2+} levels may be associated with the apoptotic process (32). Apoptosis is often accompanied by increased Ca^{2+} levels, and the addition of calcium regulators or calmodulin inhibitors can directly induce apoptosis. These results suggested that apoptosis is closely associated with intracellular Ca^{2+} (33). In the present study, LCA induced apoptosis by the release of intracellular Ca^{2+}, but not extracellular Ca^{2+}, thus suggesting that intracellular Ca^{2+} is closely associated with apoptosis in T24 cells. Our previous study demonstrated that LCA inhibited proliferation by inducing ROS production in T24 cells (31). The present study demonstrated that when intracellular Ca^{2+} was inhibited, LCA-induced apoptosis and ROS generation were suppressed. These results indicated that Ca^{2+} may act upstream of ROS generation in T24 cells (Fig. 3).

Mitochondria have been demonstrated to have a crucial role in cell apoptosis, and the mitochondria-dependent apoptotic pathway is involved in LCA-induced apoptosis (7). The present study examined whether apoptosis is mediated through mitochondrial dysfunction, and the MMP was analyzed using the mitochondrion-sensitive dye JC-1. The results indicated that LCA was capable of inducing T24 cell apoptosis by decreasing MMP (Fig. 4A). In addition, mitochondrial apoptosis-related genes Bax, Bim, Apaf-1, caspase-9 and caspase-3 were activated by LCA, whereas Bcl-xL was inhibited. These data suggested that LCA induced apoptosis through mitochondrial dysfunction (Fig. 4B and C). Furthermore, when intracellular Ca^{2+} levels were decreased, Bax, Bim, Apaf-1 caspase-9 and caspase-3 expression levels were inhibited, whereas Bcl-xL expression was not altered. These results indicated that it was the increased levels of Ca^{2+} that regulated the LCA-induced mitochondrial apoptotic pathway (Fig. 5).

Calpain is necessary for reconstruction of the cytoskeleton, intracellular signal transduction, regulation of the cell cycle, and apoptosis. Previous studies have reported that high cellular concentrations of Ca^{2+} can activate m-calpain, which can subsequently activate caspase-4, thus inducing the caspase cascade reaction and leading to ER stress-associated apoptosis (34-36). The present study, as expected, demonstrated that treatment of T24 cells with LCA induced an upregulation in the expression levels of calpain 2 and caspase-4. Conversely, calpain 2 and caspase-4 expression levels were reduced alongside a reduction in the concentration of Ca^{2+}. These findings indicated that intracellular Ca^{2+} is associated with LCA-induced ER stress-associated apoptosis in T24 cells (Figs. 6 and 7).

In conclusion, the present study demonstrated that LCA induces apoptosis in T24 cells, and its mechanism may be associated with an intracellular imbalance in calcium homeostasis. LCA was able to induce intracellular Ca^{2+} release, promote ROS accumulation, and regulate the expression of mitochondrial apoptosis-related genes (Bax, Bim, Apaf-1, caspase-9 and caspase-3 expression) and ER stress-induced apoptosis-related genes (calpain2 and caspase-4), thus resulting in apoptosis. These data provide further support for the notion that LCA should be further explored as a possible chemopreventive modality, as well as in terms of its possible effectiveness in the treatment of bladder cancer.

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