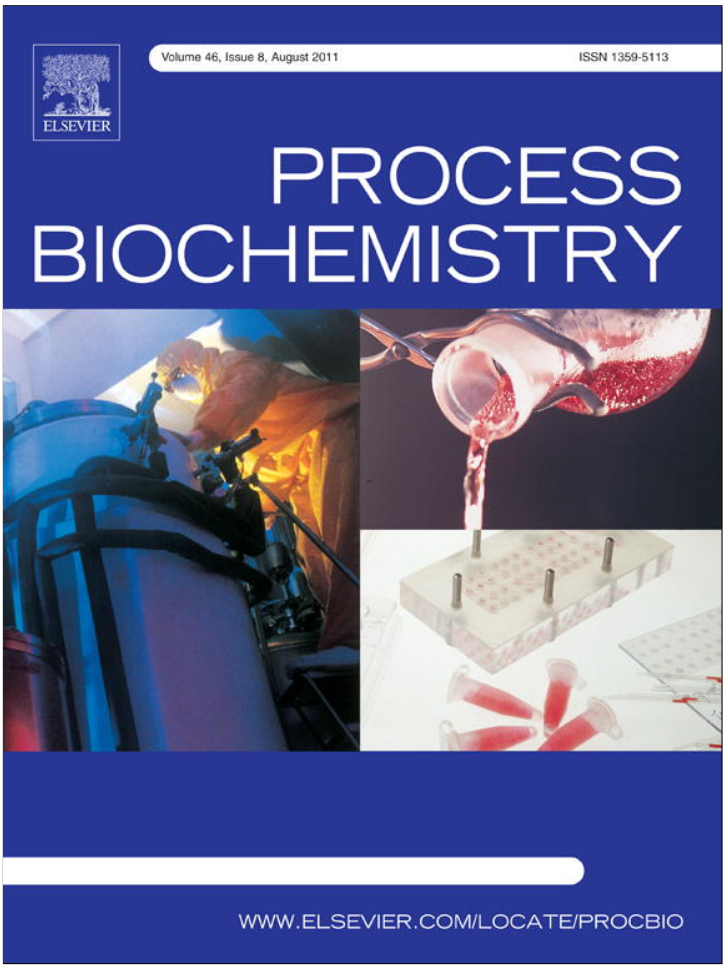

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Calvatia lilacina protein extract induces apoptosis through endoplasmic reticulum stress in human colon carcinoma cells

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ABSTRACT

In our previous study, *Calvatia lilacina*, which is an edible medicinal mushroom, was shown to have anti-cancer effects in some human colon cancer cells by inducing apoptosis. In this paper, we demonstrate the potential mechanism by which *C. lilacina* protein extract (CL) induces apoptosis through endoplasmic reticulum (ER) stress in human colon cancers. Treatment of human SW480, HT-29, and DLD-1 colon cancer cells with CL induced the formation of ER stress-related cytoplasmic vacuoles. Treatment of SW480 cells with CL also induced eukaryotic initiation factor-2alpha (eIF-2alpha) phosphorylation, cleavage of caspase-4 and -9, expression of activating transcription factor (ATF)4, ATF3, CCAAT/enhancer-binding protein-homologous protein (CHOP) and glucose-regulated protein78 (GRP78). We also showed that CL activated c-Jun NH(2)-terminal kinase (JNK) and increased cytoplasmic calcium. *N*-Acetylcysteine (NAC) or SP600125 inhibited CHOP expression in CL-treated SW480 cells, suggesting that CL-induced ER stress is dependent on intracellular glutathione (GSH) depletion and JNK activation. Pretreatment with salubrinal, SP600125, and BAPTA partially protected cells from CL-induced apoptosis. Taken together, these data suggest that the anti-cancer mechanism of CL is at least in part attributed to its ability to induce ER stress, JNK activation, and cytoplasmic calcium accumulation in human colon cancer cells.

1. Introduction

Calvatia lilacina (Mont. et Berk.) Lloyd (Fam. Lycoperdaceae) (Fig. 1) is an edible medicinal mushroom that is widely used for the treatment of throat and nasopharyngeal pain, voice loss, coughing, hemorrhagic vomiting, nose bleeding, and traumatic bleeding in China and Taiwan. Our previous study reported that *C. lilacina* protein extract (CL) has anti-cancer properties [1,2]. Treatment with CL significantly reduced cell viability in several human colon cancer lines, such as SW480, Colo250, Colo320DM, and DLD-1 cells, and in human monocytic leukemia THP-1 cells [1,2]. Treatment with CL induced many apoptosis-related events, such as mitochondrial transmembrane potential reduction, mitochondrial cytochrome c release, Bax over-expression, glutathione (GSH) depletion, and reactive oxygen species (ROS) production, in human colon cancer cell lines [1]. Among these processes, the downregulation of gamma-glutamylcysteine synthetase, which resulted in GSH depletion, is the critical mechanism to induce apoptosis [1].

The endoplasmic reticulum (ER) stress response comprises a group of adaptive pathways that can be initiated by different perturbations of normal ER function, such as lipid or glycolipid imbalances, accumulation of unfolded proteins, or changes in the ionic states of the ER lumen [3]. The primary purpose of the ER stress response is to diminish stressful disruption and regain appropriate ER homeostasis. However, under conditions of strong or continual ER stress, these pathways will induce apoptosis [3].

Some studies indicate a connection between oxidative stress and ER stress as evidenced by increased oxidative stress by tert-butyl hydroperoxide, increased accumulation of ROS in the ER, and upregulated expression of CCAAT/enhancer-binding protein-homologous protein (CHOP)/GADD153, which is a transcription factor associated with growth arrest and apoptosis in the event of prolonged ER stress [4]. ROS also act as an early event that triggers ER stress and mitochondrial apoptotic pathways in cirsimaritin-induced human gallbladder carcinoma GBC-SD cell apoptosis [5].

Abbreviations: CL, *Calvatia lilacina* protein extracts; ER, endoplasmic reticulum; eIF-2alpha, eukaryotic initiation factor-2alpha; ATF, activating transcription factor; CHOP, CCAAT/enhancer-binding protein-homologous protein; GRP78, glucose-regulated protein78; JNK, c-Jun NH(2)-terminal kinase; NAC, *N*-acetylcysteine; GSH, glutathione; ROS, reactive oxygen species; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate buffer saline; DMSO, dimethyl sulfoxide; PI, propidium iodide; UPR, unfolded protein response; IL-1beta, interleukin-1beta.

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Fig. 1. The photograph of *Calvatia lilacina*.

In addition, mitochondrial dysfunction is directly linked to ER stress response, as demonstrated by elevated levels of cytosolic free Ca^{2+} due to mitochondrial dysfunction and concomitant activation of p38-mitogen-activated protein kinase in human liver sk-HepI cells [6]. Some agents also exert ER stress to counteract human colon cancer. Treatment with resveratrol induces CHOP and glucose-regulated protein78 (GRP78) expression in HT29 human colon carcinoma cells [7]. Rottlerin, which is a compound reported to be a PKC delta-selective inhibitor, also induced apoptosis through ER stress-related phosphorylation of eukaryotic initiation factor-2alpha (eIF-2alpha) and upregulation of CHOP and GRP78 [6]. These reports suggest that ER stress plays an important role in many anti-cancer agents.

In our previous publication, our data show that CL induces GSH depletion and ROS production during apoptosis in human colon cancers [1]. Whether these events can elicit an ER stress response and induce apoptosis remains to be determined. Thus, important questions about the molecular mechanisms underlying the ER stress induced by CL remain to be answered. Although oxidative stress damage, especially GSH depletion, to human colon cancers in response to CL treatment has been studied extensively in our laboratory, ER stress induced by CL has not been reported to the best of our knowledge. To gain a better understanding of the molecular effects of CL in colon cancer cells, the aim of the present study was to determine whether CL induces ER stress, which could be related to the induction of apoptosis in colon cancer cells. We focus on the potential mechanism of ER stress-mediated apoptosis in CL-treated cells.

2. Materials and methods

2.1. Experimental material

C. lilacina was collected from the National Chiayi University, Chiayi City, Taiwan, in May 2009.

2.2. Extraction and isolation of C. lilacina proteins

C. lilacina (450–500 g) was washed twice with distilled water and then homogenized in cold extraction buffer (50 mM KH_2PO_4, 150 mM NaCl, and 1 mM EDTA, pH 7.3) at maximum speed in a Waring blender for 10 min. The water-soluble protein extract homogenate was filtered through two layers of surgical gauze, and the filtrate was centrifuged at 8900 *g* for 20 min. The supernatants were precipitated by addition of ammonium sulfate to 80% saturation, incubation at 4 °C overnight, and then centrifuged at 8900 *g* for 30 min. The protein pellets were resuspended in a phosphate buffer solution (123 mM NaCl, 10.4 mM Na_2HPO_4, and 3.16 mM KH_2PO_4, pH 7.3) (weight/volume = 1:1) and centrifuged. The supernatants were dialyzed against a 50-fold volume of phosphate buffer solution three times at 4 °C and clarified by centrifugation. The supernatants were filtered through 0.45-μm Millex filter units (Millipore) and concentrated using Amicon Ultra-4 (10 kDa cut-off, Millipore) tubes at 4000 *g* for 20 min. The 1-ml concentrate was sterilized using 0.22-μm Millex filter units (Millipore) in a laminar flow chamber and then freeze-dried to obtain a powder and stored at −80 °C as stock. A Bio-Rad protein assay reagent (Bio-Rad Lab., Hercules, CA, USA), 12% SDS-PAGE gel electrophoresis, and Coomassie Brilliant Blue staining were utilized to detect extracted proteins.

2.3. Cell lines and reagents

The human colorectal cancer cell lines, SW480 and DLD-1, were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan, ROC). The human colon cancer cell line, HT-29, was obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (South Logan, UT, USA) and Biological Industries (South Logan, UT, USA), respectively. The primary antibodies against p-eIF-2alpha, eIF-2alpha, p-ERK, ERK, CHOP, GRP78, ATF4, ATF3, caspase-4 and caspase-9 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The primary antibodies against p-JNK, JNK, p-ERK, ERK, p-p38 and p38 were acquired from Cell Signaling Technology (Boston, MA, USA). Propidium iodide (PI), DNase-free RNase A, Fluo-3 AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), dimethyl sulfoxide (DMSO), anti-actin primary antibody and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Cell culture and treatment

SW480 cells were cultured in DMEM containing 10% FBS. DLD-1 and HT-29 cells were cultured in PRMI-1640 medium containing 10% FBS. The stock solutions of CL were dissolved in the phosphate buffer saline (PBS) (136.89 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.4), and a concentration (100 μg/ml) was prepared in DMEM or RPMI-1640 medium. Control cells were incubated with a volume of PBS equal to that added in the cultures that received CL.

2.5. Analysis of cytoplasmic vacuoles derived from the ER

After CL treatment, the medium was removed from the culture dishes, cells were rinsed with HBSS, and pre-warmed staining solution was added. The cells in culture dishes were incubated for approximately 30 min at 37 °C. Cellular morphology and cytoplasmic vacuolization were examined with a light microscope. For adherent cells, the medium was removed from the culture dish, cells were rinsed with HBSS, and pre-warmed ER-Tracker™ green dyes staining solution was added. The cells were incubated for approximately 15 min at 37 °C. The staining solution was replaced with fresh probe-free medium. Cellular morphology and cytoplasmic vacuolization were examined with a light microscope and a fluorescence microscope.

2.6. Western blotting analysis

After treatment, cells were washed with PBS, resuspended in a protein extraction buffer for 10 min, and centrifuged at 12,000 *g* for 10 min at 4 °C to obtain total extracted proteins (that is, supernatant). Protein concentrations were measured with a Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA). The extracted cellular proteins were boiled in loading buffer, and an aliquot corresponding to 50 μg of protein was separated on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were electrotransferred onto a polyvinylidene fluoride transfer membrane. After blocking, the membranes were incubated with various primary antibodies overnight and then washed with a PBST solution (0.05% Tween 20 in PBS). After washing, the secondary antibody labeled with horseradish peroxidase was added to the membrane for 1 h and then washed with PBST solution (0.05% Tween 20 in PBS). The antigen–antibody complexes were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with a chemiluminescence analyzer.

2.7. Measurement of intracellular calcium by flow cytometry

Intracellular calcium was detected by flow cytometry using Fluo-3 AM. Cells were washed once with PBS, trypsinized and collected by centrifugation, and suspended in PBS. Cells were stained with Fluo-3 AM for 30 min and then treated with 100 μg/ml CL for 1000 s. Intracellular calcium levels, as indicated by the fluorescence of Fluo-3, were measured with a Becton–Dickinson FACSan flow cytometer.

2.8. Cell cycle and DNA damage analysis

The cell cycle distribution and DNA damage were evaluated with PI staining and flow cytometry. PI is a specific fluorescent dye that stains double-stranded DNA. In methanol-fixed cells, the PI molecules translocate into the nucleus and bind to the double-stranded DNA. Using flow cytometry, PI was excited at 488 nm and emitted fluorescence at 580–630 nm. The G_1 and G_2/M phases are represented by the diploid and the tetraploid regions, respectively, of the DNA distribution histograms. Cells in the S phase were found between the diploid and the tetraploid regions. The length of double-stranded DNA in cells with DNA damage was smaller than that of cells in the G_1 phase. Fewer PI molecules were found in DNA-damaged cells as compared with the G_1 phase; the fluorescent intensity of PI in DNA-damaged cells was weaker than that of cells in the G_1 phase. The percentage of DNA-damaged cells was characterized as the percentage of cells in the $SubG_1$ region of the DNA distribution histograms. Cells (1×10^6) were cultured in 60-mm tissue-culture dishes for 24 h. The culture medium was replaced with new medium, and then cells were exposed to various agents for 24 h. After treatment, adherent and floating cells were pooled, washed

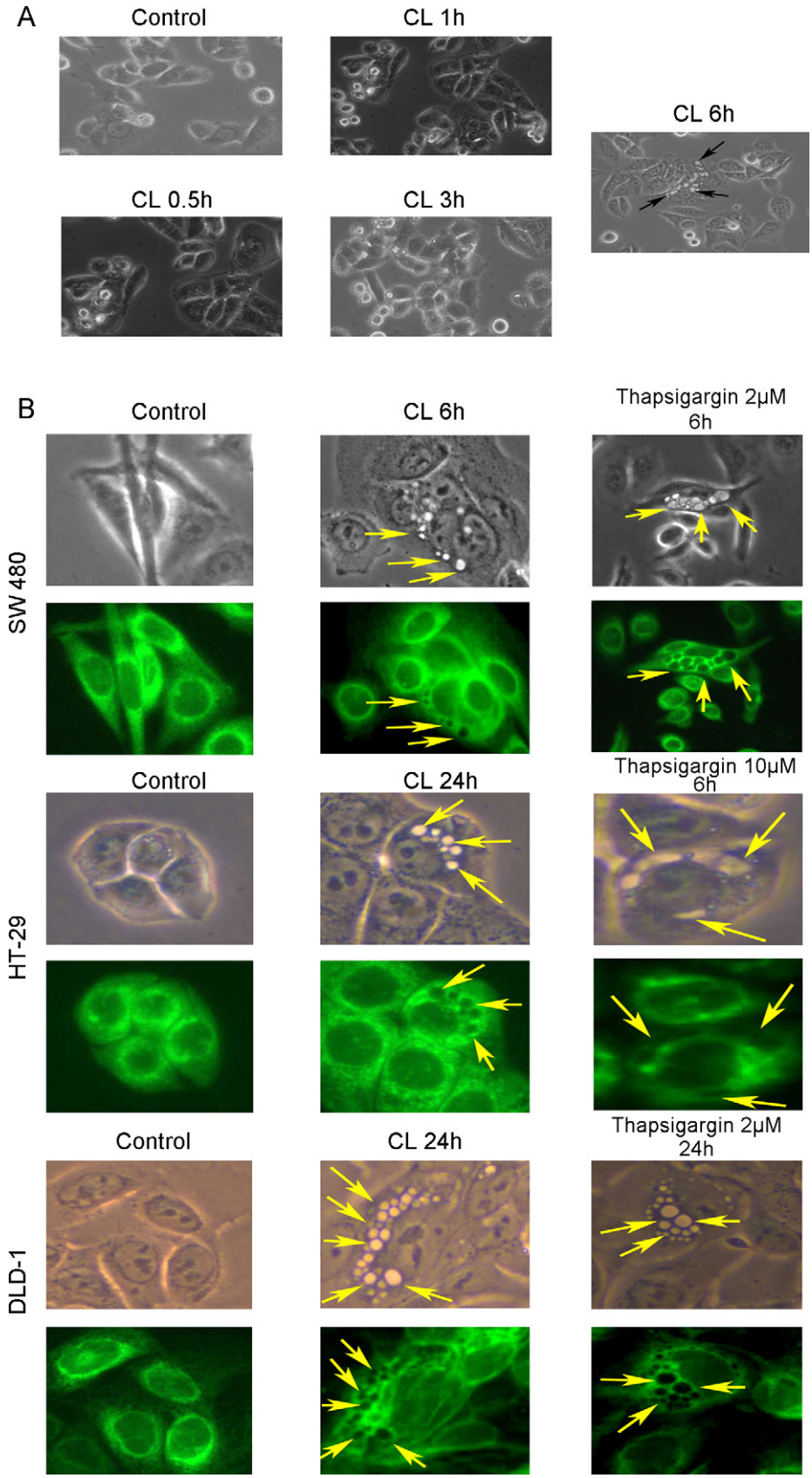

Fig. 2. CL induces early accumulation of cytoplasmic vacuoles in human colon cancer cell lines. (A) SW480 cells were treated with 100 μg/ml of CL. Phase-contrast images (original magnification, 400×) of cells were obtained after 0.5, 1, 3, and 6 h. (B) SW480, DLD-1, and HT-29 cells were treated with 100 μg/ml CL or thapsigargin (an ER stress inducer) for the indicated times and concentrations. After treatment, phase-contrast images and fluorescent images (original magnification, 400×) of cells were obtained. Fluorescent images (original magnification, 400×) of live cells were obtained after staining with the ER-Tracker™ green dye. The arrows indicate the specific cytoplasmic vacuoles associated with ER stress.

with PBS, fixed in a PBS–methanol (1:2 (volume/volume)) solution, and then maintained at 4 °C for at least 18 h. After one wash with PBS, the cell pellets were stained with a fluorescent probe solution containing PBS, 40 μg/ml PI, and 40 μg/ml DNase-free RNase A for 30 min at room temperature in the dark. The DNA fluorescence of PI-stained cells was analyzed by excitation at 488 nm and monitored through a 630/22-nm band-pass filter using a Becton–Dickinson FACScan flow cytometer (Franklin Lakes, NJ, USA). A minimum of 10,000 cells was counted per sample, and DNA histograms were evaluated further using Modfit software on a PC workstation to calculate the percentage of cells in various phases of the cell cycle.

2.9. Measurement of GSH depletion

After treatment, the SW480 cells were incubated with 25 μM CMF-DA for 20 min at 37 °C in a CO_2 incubator. Once CMF-DA inside the cell, cytosolic esterase cleavages their acetates, and then the chloromethyl group reacts with intracellular thiols, converting the probe into a cell-impermeable fluorescent dye-thioether adduct, chloromethylflourescein (CMF). The CMF fluorescence is directly related to intracellular GSH level. After CMF-DA staining, the cells were washed with PBS, collected by centrifugation, and then measured with a Becton–Dickinson FACSan flow cytometer.

2.10. Statistical analysis

Data are presented as mean ± standard deviation from at least three independent experiments and analyzed using Student's *t*-test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. CL treatment induces cytoplasmic vacuoles derived from the ER in colon cancer cells

To characterize the ER stress induced by CL treatment, we observed the morphological features of cytoplasmic vacuoles using light microscopy. As shown in Fig. 2A, the accumulation of extensive cytoplasmic vacuolization was seen at 6 h after treatment of CL in SW480 cells. To test the hypothesis that the vacuoles induced by CL are derived from the ER, we stained colon cancer cell lines, SW480, HT-29, and DLD-1, with the ER-Tracker™ green dyes, which is a cell-permeant, live-cell stain that is highly selective for the ER membrane. In control cells, the green dye was diffusely localized in the perinuclear region, which is a pattern typical for the ER (Fig. 2B). However, the ER membranes were concentrated in discrete globular structures that matched the lucent vacuoles observed by phase-contrast microscopy after CL treatment (Fig. 2B).

3.2. CL treatment induces the expression of ER stress-related proteins in SW480 cancer cells

Phosphorylation of eIF2alpha, which is a branch of the unfolded protein response (UPR), mediates the translational upregulation of selected stress-induced proteins, such as CHOP [8]. As shown in Fig. 3A, CL induced phosphorylation of eIF2alpha at 3 h and 6 h. Other UPR proteins, namely, ATF3 and ATF4, were examined to confirm the induction of ER stress by CL treatment. CL also increased the protein expression of ATF3 and ATF4 at the 3 h and 6 h (Fig. 3A). To evaluate the extent of ER stress after CL treatment, we analyzed additional indicators of ER stress. Cells were treated with CL for various lengths of time and the expression of CHOP and GRP78 was analyzed using western blotting. Fig. 3B shows that CL stimulated the two selected ER stress indicators in a similar fashion. Both CHOP and GRP78 were substantially elevated, with a prominent increase noted at 3 h and 6 h. Because caspase activation is thought to induce apoptosis, we examined whether CL mediated the activation of caspase-4 and -9 during ER stress in SW480 cells. As shown in Fig. 4, the cleavage of caspase-4 and -9 was markedly increased at 0.5 h, 1 h, 3 h, and, 6 h in CL-treated cells as compared with control cells.

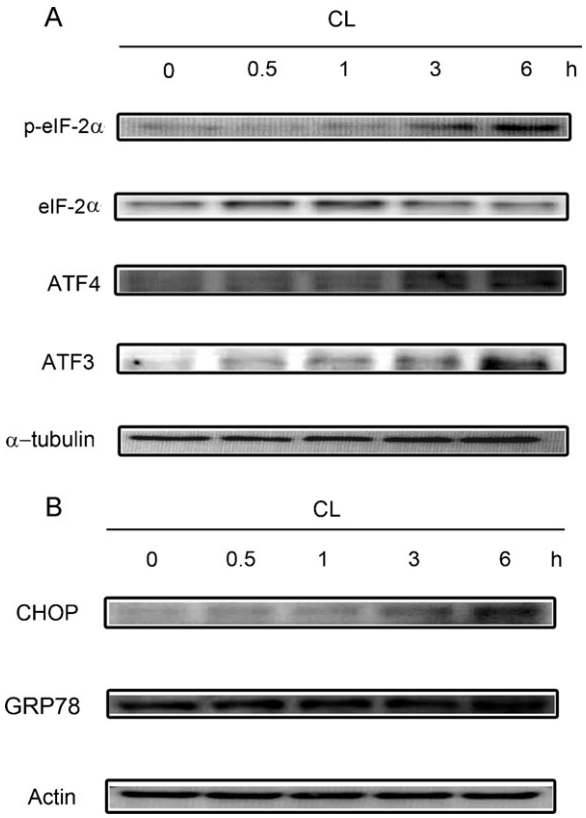

Fig. 3. Evaluation of ER stress-related proteins in CL-treated SW480 cells. Cells were plated in 60-mm culture dishes at 80% confluence and then treated with 100 μg/ml of CL for 0.5 h, 1 h, 3 h, or 6 h. After treatment, cells were washed with PBS and extracted with protein extraction buffer. Fifty micrograms of protein were loaded on a 12% SDS-polyacrylamide gel. (A) The phosphorylation of eIF2alpha and the expression of eIF2alpha, ATF4, and ATF3 were evaluated by western blotting. (B) The expression of CHOP and GRP78 were evaluated by western blotting. α-Tubulin and actin were used as internal controls. These experiments were performed at least three times; a representative experiment is presented.

3.3. CL treatment induces activation of JNK in SW480 cancer cells

Because mitogen-activated protein kinases (MAPK) activation is involved in ER stress processes [9], we further evaluated the effect of CL on MAPK activation. Exposure of SW480 cells to CL resulted in rapid and sustained phosphorylation of JNK from 0.5 h to 3 h. The phosphorylation of ERK remained unaltered by CL treatment. However, phosphorylation of p38 decreased in CL-treated SW480 cells (Fig. 5).

3.4. CL treatment transiently induces cytoplasmic calcium accumulation in SW480 cancer cells

It has been shown that treatment of various tumor cell lines with anti-cancer agents generates increased levels of intracellular calcium with subsequent activation of the endoplasmic reticulum (ER) stress response and subsequent cell death [10]. We next determined if CL could induce a cytoplasmic calcium increase. For this purpose, cells were loaded with Fluo-3 AM and exposed to CL. The increase in cytoplasmic calcium levels was measured by flow cytometry. As shown in Fig. 6, a transient increase of cytoplasmic calcium could be observed by CL treatment. Pretreatment with BAPTA for 1 h could prevent CL-induced cytoplasmic calcium accumulation in SW480 cells.

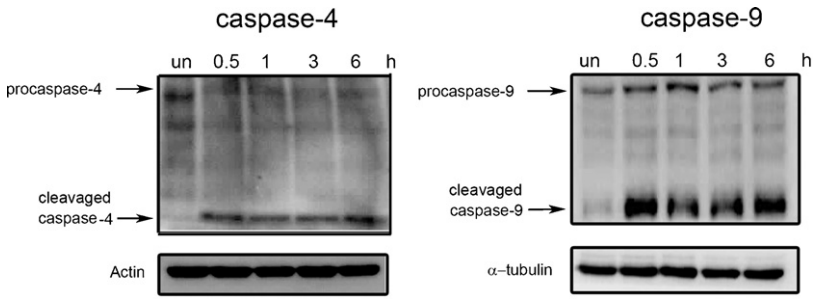

Fig. 4. Cleavages of caspase-4 and -9 in CL-treated SW480 cells. Cells were plated in 60-mm culture dishes at 80% confluence and then treated with 100 μg/ml of CL for 0.5 h, 1 h, 3 h, or 6 h. After treatment, cells were washed with PBS and extracted with protein extraction buffer. Fifty micrograms of protein were loaded on a 12% SDS-polyacrylamide gel, and the levels of procaspase-4 and -9 and cleavages of caspase-4 and -9 were evaluated using western blotting. α-Tubulin and actin were used as internal controls. These experiments were performed at least three times; a representative experiment is presented.

3.5. CL-induced ER stress is dependent on GSH depletion and JNK activation in SW480 cancer cells

Our previous study demonstrated that CL treatment induced GSH depletion [1]. To evaluate whether GSH depletion, JNK activation, and cytoplasmic calcium accumulation are involved in CL-induced ER stress in SW480 cells, several agents were employed in the study: first, *N*-acetylcysteine (NAC), an intracellular GSH synthetic agent, was used to investigate the role of GSH depletion. Second, SP600125, a JNK inhibitor, was used to investigate the role of JNK activation pathway. Third, BAPTA, an intracellular calcium chelator, was used to investigate the role of cytoplasmic calcium accumulation. SW480 cells were pretreated with NAC, SP600125, or BAPTA for 1 h and then treated with CL for 6 h. The expression of CHOP was evaluated using western blotting. As shown in Fig. 7A, pretreatment with NAC or SP600125 decreased the expression of CHOP in CL-treated cells. The expression of GRP78 and caspase-4 was also partially inhibited by NAC or SP600125 pretreatment. In addition, pretreatment with NAC or SP600125 for 1 h could prevent CL-induced GSH depletion or phosphorylation of JNK in SW480 cells, respectively (Fig. 7B and C). These results suggest that CL-induced ER stress is dependent on GSH depletion and JNK activation. On the other hand, pretreatment with BAPTA did not decrease the expression of CHOP in CL-treated cells, suggesting cytoplasmic calcium accumulation may not be a critical role in CL-induced ER stress.

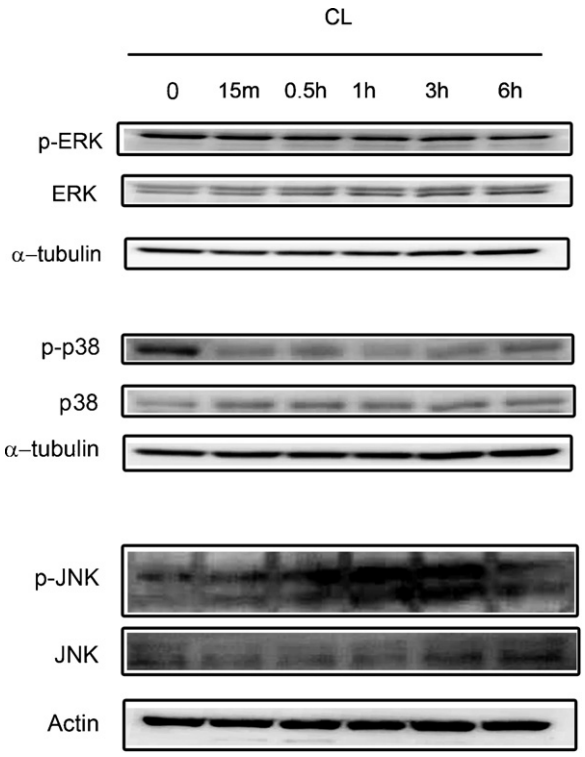

Fig. 5. Evaluation of JNK, ERK and p38 activation in CL-treated SW480 cells. Cells were plated in 60-mm culture dishes at 80% confluence and then treated with 100 μg/ml of CL for 15 min, 0.5 h, 1 h, 3 h, and 6 h. After treatment, cells were washed with PBS and extracted with protein extraction buffer to obtain total protein. Fifty micrograms of protein were loaded on a 12% SDS-polyacrylamide gel. Phosphorylated JNK, ERK and p38 and non-phosphorylated JNK, ERK and p38 were evaluated using western blotting. α-Tubulin and actin were used as internal controls. These experiments were performed at least three times; a representative experiment is presented.

3.6. ER stress, JNK activation, and cytoplasmic calcium accumulation play important roles in CL-induced apoptosis in SW480 cancer cells

To evaluate whether ER stress, JNK activation, and cytoplasmic calcium accumulation are involved in CL-induced apoptosis, several agents were employed in the study. First, salubrinal, an ER stress inhibitor, was used to investigate the role of the ER stress pathway. Second, SP600125, a JNK inhibitor, was used to investigate the role of the JNK activation pathway. Third, BAPTA, an intracellular calcium chelator, was used to investigate the role of the cytoplasmic calcium accumulation. SW480 cells were treated with these agents for 1 h before the addition of CL. After CL treatment for 24 h, cells were collected and apoptosis was assayed by flow cytometry. As shown in Fig. 8, there were 45.8% apoptotic cells after treatment with CL for 24 h as compared with 2.9% apoptotic cells among control cells. Salubrinal pretreatment moderately inhibited CL-induced apoptosis; the percentage of apoptotic cells decreased to 22.7%. SP600125 and BAPTA pretreatment displayed a marked inhibitory effect, as the percentage of apoptotic cells was 18.7% and 19.7%, respectively. These results suggest that ER stress, JNK activation, and increases in cytoplasmic calcium play important roles in CL-induced apoptosis in SW480 cells.

4. Discussion

Our previous study demonstrated that the percentages of apoptosis were 11.8%, 43.8%, and 50.1% in SW480 cells treatment with 50, 75, and 150 μg/ml of CL, respectively [1]. It indicates that the concentration of 75–150 μg/ml of CL can induce marked apoptosis. For the reason, we selected the concentration of 100 μg/ml to induce ER stress or apoptosis in three human colon carcinoma cell lines. Damage to or stress in the ER has been shown to be capable of triggering apoptosis [11,12]. Studies have shown that unfolded protein response is responsible for ER-mediated apoptosis [12]. ER stress has been demonstrated to cause the activation of certain cas-

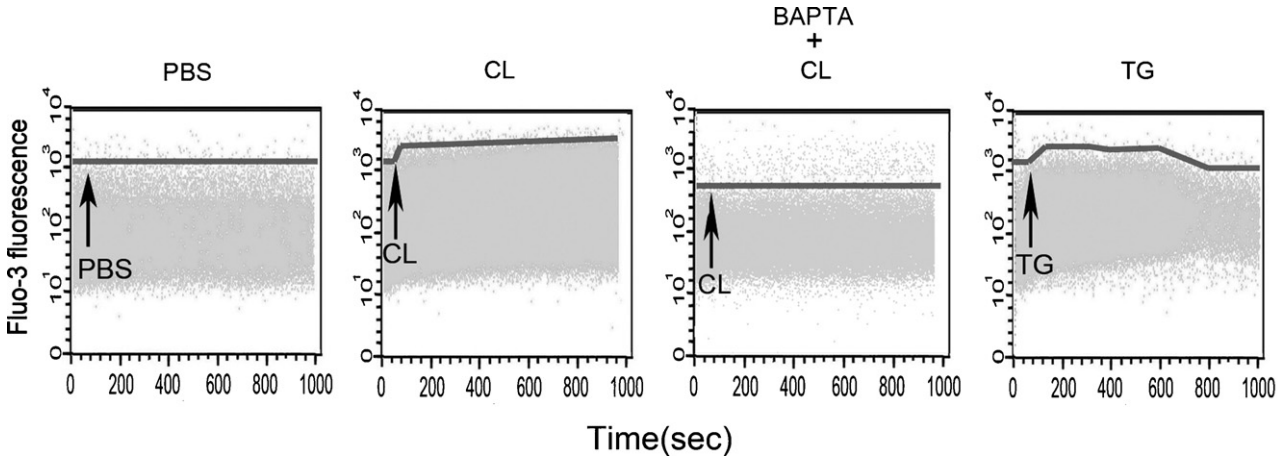

Fig. 6. Evaluation of cytoplasmic calcium in CL-treated SW480 cells. Cells were stained with 1 μM Fluo-3 AM for 30 min and then treated with PBS, 100 μg/ml of CL or 2 μM of thapsigargin (TG) for the indicated times. In the BAPTA + CL group, cells were pretreated with 10 μM BAPTA for 1 h followed by the treatment of 100 μg/ml CL for the indicated times. The intracellular fluorescence of Fluo-3 was measured with a Becton–Dickinson FACScan flow cytometer. The arrows represent the initial treated time point of PBS, CL or TG. These experiments were performed at least three times; a representative experiment is presented.

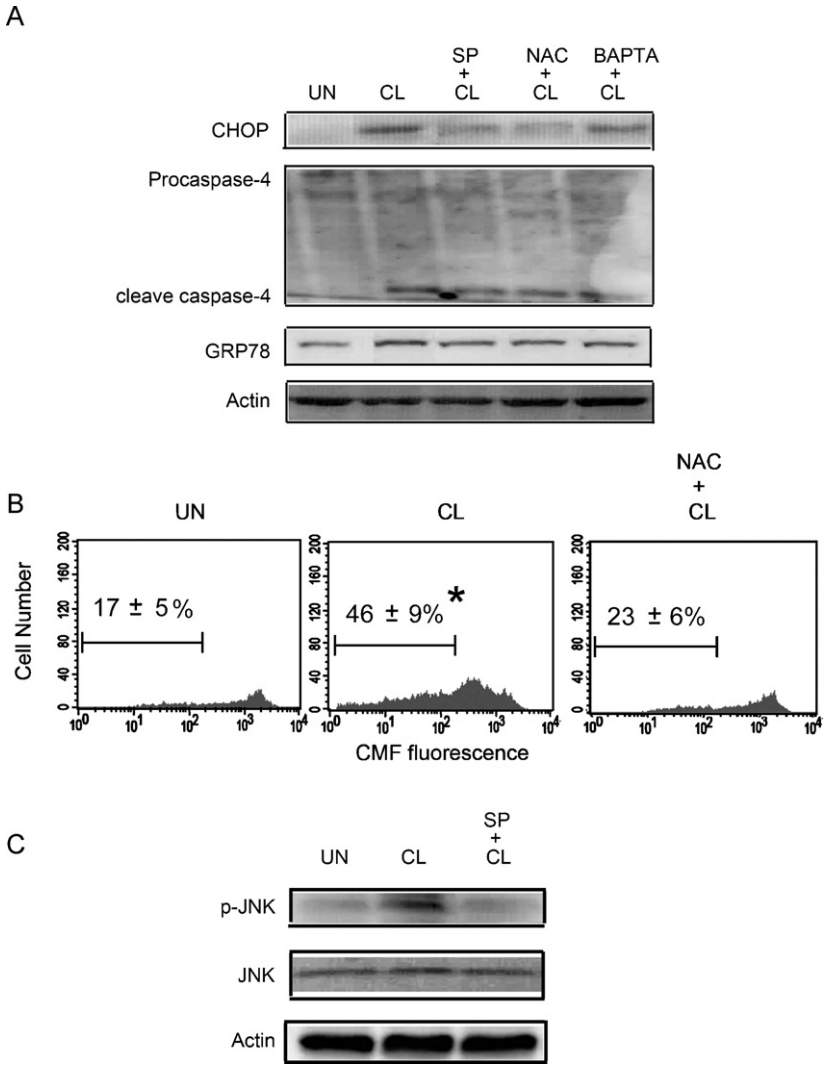

Fig. 7. Evaluation of critical events in CL-induced ER stress. (A) and (C) SW480 cells were treated with 0 (control) or 100 μg/ml of CL for 6 h or pretreated with 100 μM SP600125 (SP), 10 mM *N*-acetylcysteine (NAC), or 10 μM BAPTA for 1 h, followed by 100 μg/ml of CL treatment for 6 h. After treatment, cells were washed with PBS and extracted with protein extraction buffer to obtain total protein. Fifty micrograms of protein were loaded on a 12% SDS-polyacrylamide gel and the expression of CHOP, caspase-4, GRP78, and phosphorylation of JNK were evaluated by western blotting analysis. (B) SW480 cells were treated with 0 (control) or 100 μg/ml of CL for 6 h or pretreated with 10 mM *N*-acetylcysteine (NAC) for 1 h, followed by 100 μg/ml of CL treatment for 6 h. After treatment, the cells were incubated with 25 μM CMF-DA for 30 min in a 37 °C CO_2 incubator and then measured with a flow cytometer. These experiments were performed at least three times; a representative experiment is presented.

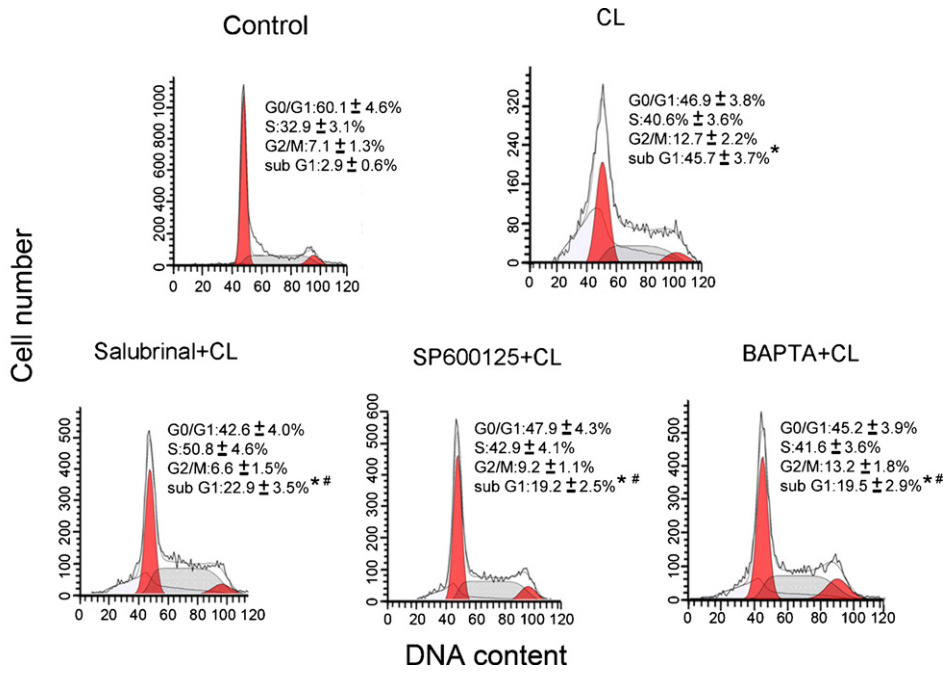

Fig. 8. ER stress, JNK activation, and cytoplasmic calcium accumulation are involved in CL-induced apoptosis. SW480 cells were treated with 0 (control) or 100 μg/ml of CL alone for 24 h or pretreated with 100 μM salubrinal, 100 μM SP600125 (SP), or 10 μM BAPTA for 1 h, followed by 100 μg/ml of CL treatment for 24 h. All samples were subsequently processed for cell cycle analysis. Data of apoptotic cells (Sub G1) in each panel presents as mean ± standard deviation from at least three independent experiments and analyzed statistically. Significant differences for the untreated group and CL group are $P < 0.05$ (*) and $P < 0.05$ (#), respectively.

pases, such as caspase-4, and the induction of apoptosis in human cells [13]. In this study, we demonstrated that treatment with CL can activate caspase-4 and caspase-9 and results in apoptosis in SW480 cells, suggesting that the ER stress-related caspase cascade is involved in CL-induced apoptosis.

In response to ER stresses, a family of protein kinases phosphorylate eIF2 (with eukaryotic initiation factor 2) can be used to diminish cellular injury or alternatively cause apoptosis. Phosphorylation of eIF2alpha decreases global translation, allowing cells to preserve resources and to regulate gene expression to operate effectively under stress conditions. eIF2 phosphorylation induces translation of specific mRNAs, such as the basic leucine zipper transcriptional regulator activating transcription factor (ATF)4, which enhances the expression of additional transcription factors, ATF3 and CHOP/GADD153 (that is, growth arrest and DNA-damage-inducible protein), which assist in the regulation of genes involved in apoptosis, redox, and metabolism [8]. Additional stress pathways, such as those regulated by MAPK pathways, can contribute to the activity of CHOP and ATF3 [8]. In this study, we demonstrate that treatment with CL can phosphorylate JNK and eIF2alpha, which subsequently upregulates ATF4, ATF3, and CHOP, resulting in an ER stress response. We also demonstrate that pretreatment with salubrinal, an eIF2alpha inhibitor, partially prevented the level of apoptosis in SW480 cells (Fig. 8). These data suggest that CL-induced ER stress is involved in the apoptosis pathway.

Perturbation of ER calcium homeostasis is one of the early events leading to disruption of ER functions. During ER stress or ER damage, calcium released from the ER to the cytosol is an important signaling element in apoptosis induced by many anti-cancer agents [14]. In addition, increased cytosolic calcium seems to be a result of ER calcium depletion through a process known as capacitative calcium entry [15]. In our study, a transient increase in cytosolic calcium level was observed in cells treated with CL (Fig. 6), however, pretreatment with BAPTA did not inhibit the expression of CHOP (Fig. 7), indicating that cytoplasmic calcium may not a critical role in CL-induced ER stress in SW480 human colon cancer cells.

Several recent reports have indicated that treatment of cells with buthionine sulfoximine (BSO), which is an intracellular GSH-depletion agent, leads to the activation of the ER stress [16]. BSO can induce obvious ER stress as demonstrated by upregulation of BiP, calregulin, phosphorylation of IRE1alpha and eIF2alpha without affecting total IRE1alpha and eIF2alpha. BSO treatment led to marked apoptosis, increased expression of CHOP/GADD153 and Bax, activated caspase-12 and caspase-3, and reduced Bcl-2 expression and phosphorylation of JNK [16]. The formation of large intracellular vesicles, which coincided with increased gene expression of the activating transcription factor 6, which is a transducer of the ER stress response, was prominent in response to the BSO treatment [17]. In line with these observations, our data indicate that NAC pretreatment inhibits CL-induced CHOP production (Fig. 7). Furthermore, our previous data also demonstrate that NAC prevents the apoptosis level in CL-treated SW480 colon cancer cells [1]. This finding fits well with the recent observation that NAC improves arsenite-induced apoptosis by eradicating the ER stress pathway. In addition, arsenite-induced reductions in procaspase-12 and increases in caspase-12, which is an ER-specific enzyme, were prevented after NAC treatment, indicating that NAC inhibits arsenite-induced toxicity by inhibiting ER stress [18]. Taken together, these results clearly indicate that GSH depletion is crucial role for ER stress during CL treatment in SW480 colon cancer cells.

Another apoptotic pathway in ER stress involves the activation of the JNK pathway, which may contribute to apoptosis in response to other stimuli [19]. For example, the proinflammatory cytokine interleukin-1beta (IL-1beta) is a significant determinant of pancreatic apoptosis and cell death, which are common characteristics during diabetes. IL-1beta treatment was accompanied by a marked increase in JNK phosphorylation with increased levels of the ER stress markers CHOP and ATF4. IL-1beta also led to increased phosphorylation of eIF2alpha. All of these events could be prevented by pretreatment with the JNK inhibitor, SP600125 [20]. Similar to our present results, we demonstrated that pretreatment with SP600125 partially inhibited the CL-induced CHOP, indicat-

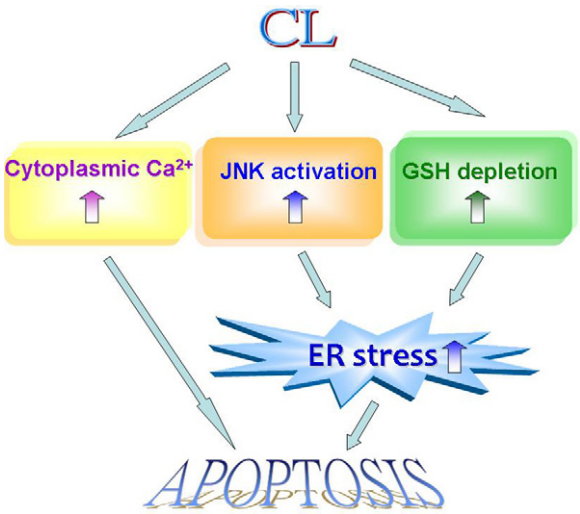

Fig. 9. Schematic illustration of a proposed model of CL-induced ER stress and apoptosis in colon cancer cells.

ing that JNK activation plays an important role in CL-induced ER stress. Other studies also indicate that some anticancer agents, such as fenretinide and docetaxel, induce an apoptotic response that is characterized by JNK activation and ER stress [21,22]. In this study, western blotting analysis showed that treatment with CL enhanced JNK activation in a time-dependent manner, and SP600125 partially inhibited CL-induced apoptosis, suggesting that JNK activation is a critical step in CL-induced apoptosis in human colon cancer cells.

Our previous study has demonstrated that the major proteins with various molecular weights around 70, 55, 45, 40, 28, 24, 20, 18 and 11 kDa were visualized after Coomassie Brilliant Blue staining in 36 and 48 μg of protein extracts of CL [2]. In our previous study, CL could induce mitochondrial damage in human colon cancer SW480 cell line [1]. We have demonstrated that CL could decrease the mitochondrial transmembrane potential from 3 to 24 h and induce cytochrome *c* release to cytosol from 3 to 12 h in SW480 cells [1]. CL also increased the Bax expression 6 h after the treatment of SW 480 cells [1]. Mitochondrial damage is another important event to induce apoptosis. These results suggest that CL-induced apoptosis is multiple pathways. Our previous study also demonstrated that CL markedly decreased the gamma-GCS expression as early as 0.5 h after treatment of SW480 cells [1]. The gamma-GCS expression was less than 50% after 3 and 6 h of CL treatment. The percentage of intracellular GSH depletion cells also significantly increased after 3, 6, 12, and 24 h of treatment, as compared with untreated cells [1]. It suggests that the inhibition of gamma-GCS expression is related GSH depletion in CL treatment.

5. Conclusions

Our studies demonstrate for the first time that CL causes apoptosis in human colon cancer cells via multiple pathways, including ER stress, JNK activation and cytoplasmic calcium accumulation. Furthermore, GSH depletion and activation of JNK are two crucial steps to provoke ER stress in CL-treated colon cancer cells. A hypothetical diagram that illustrates the activation of ER stress and apoptotic effects of CL in human colon cancer cells is depicted in Fig. 9. Our study suggests a molecular target of CL, which in turn provides a better understanding of the potential of CL to counteract colon cancer. CL might serve as a potential chemopreventative agent or food supplement for human colon cancer patients.

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References

[1] Tsay JG, Chung KT, Yeh CH, Chen WL, Chen CH, Lin MHC, et al. *Calvatia lilacina* protein-extract induces apoptosis through glutathione depletion in human colorectal carcinoma cells. J Agric Food Chem 2009;57:1579–88.
[2] Wu JY, Chen CH, Chang WH, Chung KT, Liu YW, Lu FJ, et al. Anti-cancer effects of protein extracts from *Calvatia lilacina*, *Pleurotus ostreatus*, and *Volvariella volvacea*. eCAM 2010, doi:10.1093/ecam/neq057.
[3] Kardosh A, Golden EB, Pyrko P, Uddin J, Hofman FM, Chen TC, et al. Aggravated endoplasmic reticulum stress as a basis for enhanced glioblastoma cell killing by bortezomib in combination with celecoxib or its non-coxib analogue, 2,5-dimethyl-celecoxib. Cancer Res 2008;68:843–51.
[4] He S, Yaung J, Kim YH, Barron E, Ryan SJ, Hinton DR. Endoplasmic reticulum stress induced by oxidative stress in retinal pigment epithelial cells. Graefes Arch Clin Exp Ophthalmol 2008;246:677–83.
[5] Quan Z, Gu J, Dong P, Lu J, Wu X, Wu W, et al. Reactive oxygen species-mediated endoplasmic reticulum stress and mitochondrial dysfunction contribute to cirsimaritin-induced apoptosis in human gallbladder carcinoma GBC-SD cells. Cancer Lett 2010;295:252–9.
[6] Lim JH, Lee HJ, Ho JM, Song J. Coupling mitochondrial dysfunction to endoplasmic reticulum stress response: a molecular mechanism leading to hepatic insulin resistance. Cell Signal 2009;21:169–77.
[7] Um HJ, Bae JH, Park JW, Suh H, Jeong NY, Yoo YH, et al. Differential effects of resveratrol and novel resveratrol derivative, HS-1793, on endoplasmic reticulum stress-mediated apoptosis and Akt inactivation. Int J Oncol 2010;36:1007–13.
[8] Wek RC, Jiang HY, Anthony TG. Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans 2006;34:7–11.
[9] Zou CG, Cao XZ, Zhao YS, Gao SY, Li SD, Liu XY, et al. The molecular mechanism of endoplasmic reticulum stress-induced apoptosis in PC-12 neuronal cells: the protective effect of insulin-like growth factor I. Endocrinology 2009;150:277–85.
[10] Pyrko P, Kardosh A, Liu YT, Soriano N, Xiong W, Chow RH, et al. Calcium-activated endoplasmic reticulum stress as a major component of tumor cell death induced by 2,5-dimethyl-celecoxib, a non-coxib analogue of celecoxib. Mol Cancer Ther 2007;6:1262–75.
[11] Herr I, Debatin KM. Cellular stress response and apoptosis in cancer therapy. Blood 2001;98:2603–14.
[12] Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 2003;22:8608–18.
[13] Lee do Y, Lee KS, Lee HJ, Kim do H, Noh YH, Yu K, et al. Activation of PERK signaling attenuates Abeta-mediated ER stress. PLoS One 2010;5:e10489.
[14] Strappazzon F, Torch S, Chatellard-Causse C, Petiot A, Thibert C, Blot B, et al. Alix is involved in caspase 9 activation during calcium-induced apoptosis. Biochem Biophys Res Commun 2010;397:64–9.
[15] Lu F, Tian Z, Zhang W, Zhao Y, Bai S, Ren H, et al. Calcium-sensing receptors induce apoptosis in rat cardiomyocytes via the endo(sarco)plasmic reticulum pathway during hypoxia/reoxygenation. Basic Clin Pharmacol Toxicol 2010;106:396–405.
[16] Guo R, Ma H, Gao F, Zhong L, Ren J. Metallothionein alleviates oxidative stress-induced endoplasmic reticulum stress and myocardial dysfunction. J Mol Cell Cardiol 2009;47:228–37.
[17] Todorčević M, Skugor S, Ruyter B. Alterations in oxidative stress status modulate terminal differentiation in Atlantic salmon adipocytes cultivated in media rich in n-3 fatty acids. Comp Biochem Physiol B Biochem Mol Biol 2010;156:309–18.
[18] Chao PL, Fan SF, Chou YH, Lin AM. *N*-Acetylcysteine attenuates arsenite-induced oxidative injury in dorsal root ganglion explants. Ann N Y Acad Sci 2007;1122:276–88.
[19] Oh SH, Lim SC. Endoplasmic reticulum stress-mediated autophagy/apoptosis induced by capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin is regulated by the extent of c-Jun NH2-terminal kinase/extracellular signal-regulated kinase activation in WI38 lung epithelial fibroblast cells. J Pharmacol Exp Ther 2009;329:112–22.
[20] Verma G, Datta M. IL-1beta induces ER stress in a JNK dependent manner that determines cell death in human pancreatic epithelial MIA PaCa-2 cells. Apoptosis 2010;15:864–76.
[21] Mhaidat NM, Thorne R, Zhang XD, Hersey P. Involvement of endoplasmic reticulum stress in Docetaxel-induced JNK-dependent apoptosis of human melanoma. Apoptosis 2008;13:1505–12.
[22] Appierto V, Tiberio P, Villani MG, Cavadini E, Formelli F. PLAB induction in fenretinide-induced apoptosis of ovarian cancer cells occurs via a ROS-dependent mechanism involving ER stress and JNK activation. Carcinogenesis 2009;30:824–31.