GADD153 Mediates Berberine-induced Apoptosis in Human Cervical Cancer Ca Ski Cells

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Abstract. *Berberine, an isoquinoline alkaloid, has been shown to possess anticancer properties in some cancer cell lines. Here, we report that in vitro treatment of cervical cancer Ca Ski cells with berberine decreased the percentage of viable Ca Ski cells in a dose-dependent and time-dependent manner. Berberine enhanced the apoptosis of Ca Ski cells with the induction of a higher ratio of p53 and Bax/Bcl-2 proteins, increased levels of reactive oxygen species (ROS) and Ca^{2+}, disruption of the mitochondrial membrane potential, and promotion of caspase-3 activity. In CaSki cells pretreated with the pan-caspase inhibitor z-VAD-fmk, the berberine-induced caspase-3 activity and apoptosis were significantly blocked as confirmed by flow cytometric analysis. Western blot also showed that berberine induced the expression of GADD153, a transcription factor involved in apoptosis. Thus berberine increased ROS levels leading to endoplasmic reticulum (ER) stress based on the increase of GADD153 and shown by Ca^{2+} release from the ER. When the Ca Ski cells were pretreated with catalase, GADD153 production was abrogated and apoptosis was significantly reduced.*

Life expectancy, individual genetics, changes in environmental conditions, dietary habits and lifestyle are all associated with cancer incidence. Cancer is a major cause of death in humans, and cervical cancer represents the most common malignant neoplasm in women throughout the world. There are approximately 500,000 cases per year of cervical cancer worldwide and 250,000 deaths are attributed to cervical cancer, 80% occurring in low-resource countries (1). Based on reports from the People's Health Bureau of Taiwan, about 9 people per 100 thousand die per year of cervical cancer in Taiwan. The therapy for cervical cancer is surgery, radiotherapy and chemotherapy, however, the therapy is not satisfactory. Thus cervical malignancies currently are one of the major burdens on public health and healthcare expenditures in Taiwan. There has been considerable interest in the use of phytochemicals for the prevention of cervical disorders including cervical cancer.

It is well-known that people have long used phytochemicals for the prevention of cancer. Interestingly out of 121 prescription drugs for cancer treatment, 90 are derived from natural plant sources and about 74% of these chemotherapeutic drugs were discovered by investigating a folklore claim (2, 3). Berberine is an isoquinoline alkaloid present in the roots, rhizome and stem bark of *Berberis aquifolium*, *B. vulgaris*, *B. aristata* and *Tinospora cordifolia*, and has been shown to have many biological activities such as antidiarrheal, antiarrhythmic, antimicrobial and antitumor activities (4-7). Berberine inhibits cyclooxygenase-2 transcriptional activity, DNA topoisomerase I and II and *N*-acetyltransferase activity in human cancer cell lines (8-11). Recently, in our laboratory we have also found that berberine induced G2/M arrest and apoptosis in human leukemia HL-60 cells through the mitochondria- and caspase-3 dependent pathway (12). Therefore, in an effort to develop an effective chemotherapeutic drug or agent for the prevention of cervical cancer, we attempted for the first time to examine the chemotherapeutic effect of berberine on human cervical cancer Ca Ski cells *in vitro*.

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Key Words: GADD153, apoptosis, berberine, Bcl-2, Bax, human cervical cancer, CaSki cells.

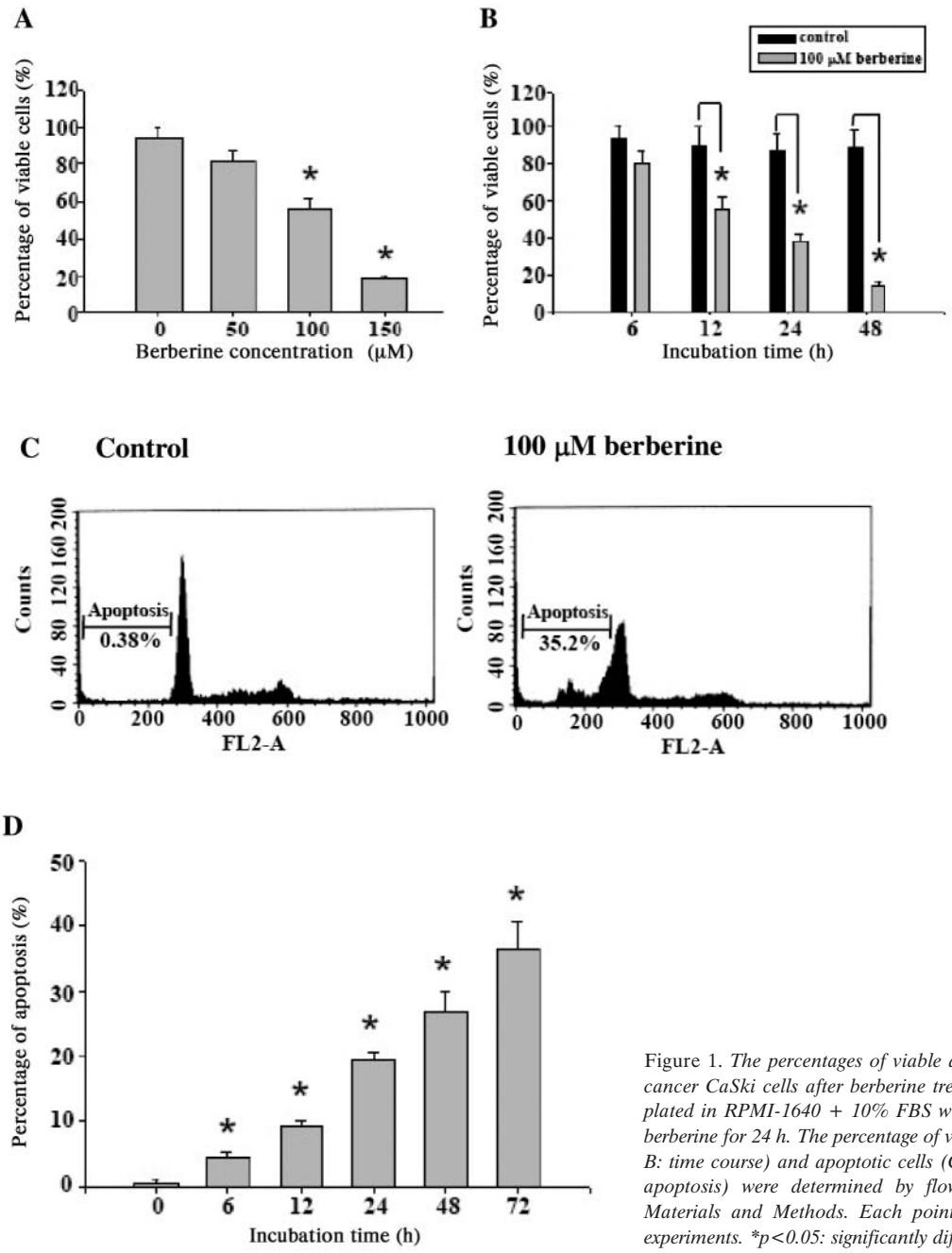

Figure 1. *The percentages of viable and apoptotic human cervical cancer CaSki cells after berberine treatment. The CaSki cells were plated in RPMI-1640 + 10% FBS with different concentrations of berberine for 24 h. The percentage of viable cells (A: dose-dependent; B: time course) and apoptotic cells (C: representative profile; D: % apoptosis) were determined by flow cytometry as described in Materials and Methods. Each point is the mean±S.D. of three experiments. *p<0.05: significantly different from the control.*

Materials and Methods

Chemicals and reagents. Berberine, propidium iodide (PI), RNase, trypan blue, Tris-HCl and triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BAPTA (a chelator of Ca^{2+}), potassium phosphates and dimethyl sulfoxide (DMSO) were obtained from Merck Co. (Darmstadt, Germany). RPMI-1640, penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS), and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). The caspase-3 activity assay kit was bought from OncoImmunin, Inc (Gaithersburg, MD, USA).

Human cervical epidermoid carcinoma cell line (CaSki). The CaSki cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75 cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 and 95% air at 1 atmosphere in RPMI-1640 medium supplemented with 10% FBS containing 1% penicillin-streptomycin (10 μg/ml penicillin and 10 μg/ml streptomycin) with

1% glutamine. The CaSki cells were cultured for several generations and checked for viability of each generation.

Flow cytometry

Analysis for cell viability. The CaSki cells were cultured in 12-well plates at a density of $2x10^5$ cells/well and grown for 24 h. Various concentrations (0, 50, 100 and 150 μM) of berberine were added or DMSO (solvent) alone for the control regimen and the cells were grown at 37˚C, 5% CO_2 and 95% air for 24 h or the cells were treated with 100 μM berberine and cultured for 6, 12, 24 and 48 h. Cell viability of the harvested cells was determined by using the flow cytometric assay as described previously (13).

Analysis of DNA content for apoptosis. Approximately $2x10^5$ cells/well of CaSki cells in 12-well plates with 100 μM berberine were incubated for 6, 12, 24, 48 or 72 h. The cells were harvested by centrifugation (1500 rpm at 15 min), and washed twice with phosphate-buffered saline (PBS), then gently fixed (drop by drop) in 70% ethanol for at least 2 h at –20˚C. The cells were then washed with PBS, incubated with 1 ml of PBS containing 0.5 μg/ml RNase A and 0.5% Triton X-100 for 30 minutes at 37˚C in the dark, and then stained with 50 μg/ml PI. The stained cells were analyzed by a FACScan laser flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an argon ion laser at 488 nm wave-length and connected to ModFit LT cell cycle analysis software (Verity Software, Topsham, ME, USA) (14).

Analysis of caspase-3 activity. Approximately $5x10^5$ cells/well of CaSki cells in 12-well plates with 0, 50, 100, and 150 μM of berberine were incubated for 24 h. The same similarly plated cells were pretreated with the broad-spectrum caspase inhibitor (z-VAD-fmk) at 50 μM for 3 h then 100 μM berberine was added and the cells were then incubated for 24 h. The cells were harvested by centrifugation (1500 rpm at 5 min). Fifty μl of 10 μM substrate solution (PhiPhiLux, a unique class of substrates for caspase-3) (OncoImmunin, Inc) were then added to each cell pellet ($5x10^5$ cells per sample) and the cells were incubated at 37˚C for 1 h. PhiPhiLux can penetrate into the cell nucleus and is converted to the fluorescent form when it is cleaved by the protease activity of caspase-3. The cells were washed once by adding 1 ml of ice cold PBS and re-suspended in fresh 1 ml PBS. The cells were analyzed with a flow-cytometer (Becton-Dickinson) and the caspase-3 activity was determined and analyzed as described previously (13).

Analysis of reactive oxygen species (ROS). Approximately $2x10^5$ cells/well of CaSki cells in 12-well plates with 0, 50 100 and 150 μM of berberine were incubated for 2 h. The cells were harvested, washed twice with PBS, re-suspended in 500 ml of 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) (10 μM), incubated at 37˚C for 30 min and then the levels of ROS were analyzed by flow cytometry as described previously (13).

Analysis of the production of Ca^{2+}. Approximately $2x10^5$ cells/well of CaSki cells in 12-well plates with or without pretreatment with BAPTA then treated with 0, 50 100 and 150 μM berberine and incubated for 24 h. The cells were harvested and washed twice. Half of the cells were used for apoptosis analysis and the other half were re-suspensed in 1-[2-amino-5-(6-carboxyindol-2-yl) phenoxy] -2-(2'-amino-5'-methylphenoxy) ethane-*N,N,N',N'* -tetra acetic acid pentaacetoxymethyl ester

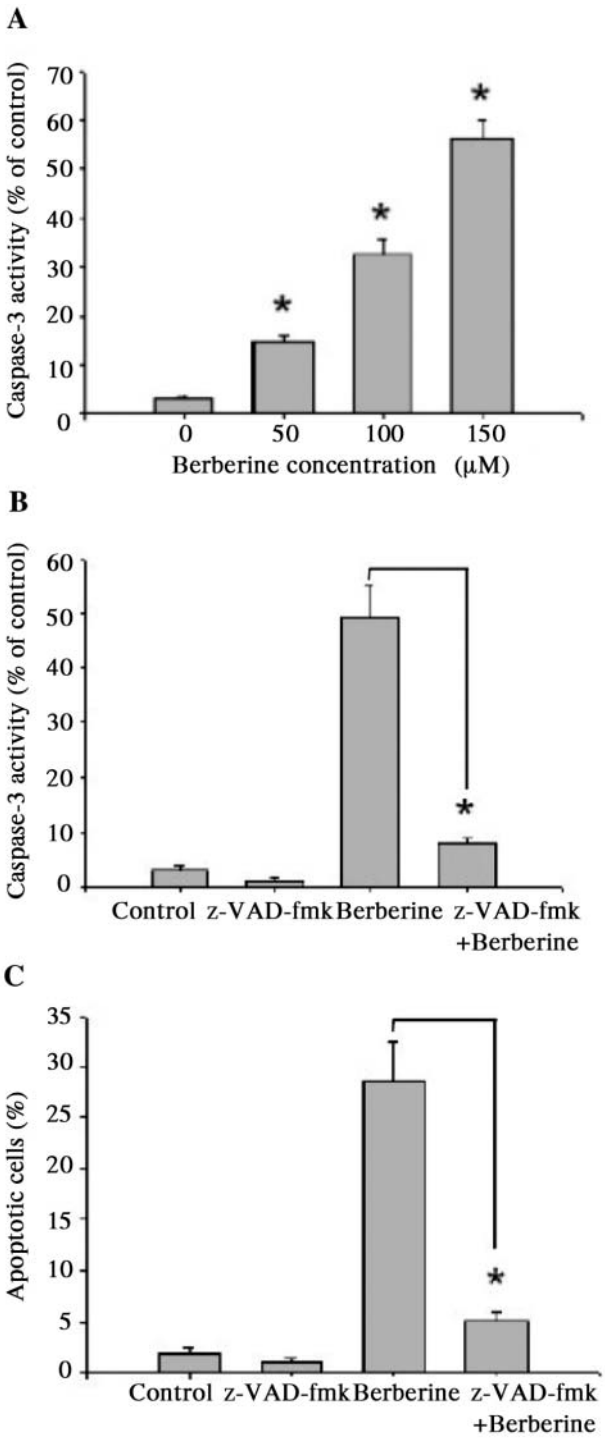

Figure 2. *Flow cytometric assays of the effects of berberine on caspase-3 activity and apoptosis from human cervical cancer CaSki cells. The Ca Ski cells were incubated with various concentrations of berberine (0, 50, 100 and 150 μM) for 24 h and caspase-3 activity was affected in a dose-dependent manner (A). The cells were pretreated with the caspase inhibitor (z-VAD-fmk) followed by 100 μM berberine for caspase-3 activity (B) and apoptosis determination (C) as described in Materials and Methods. Data represent mean ± S.D. of three experiments.*p<0.05: significantly different from the control.*

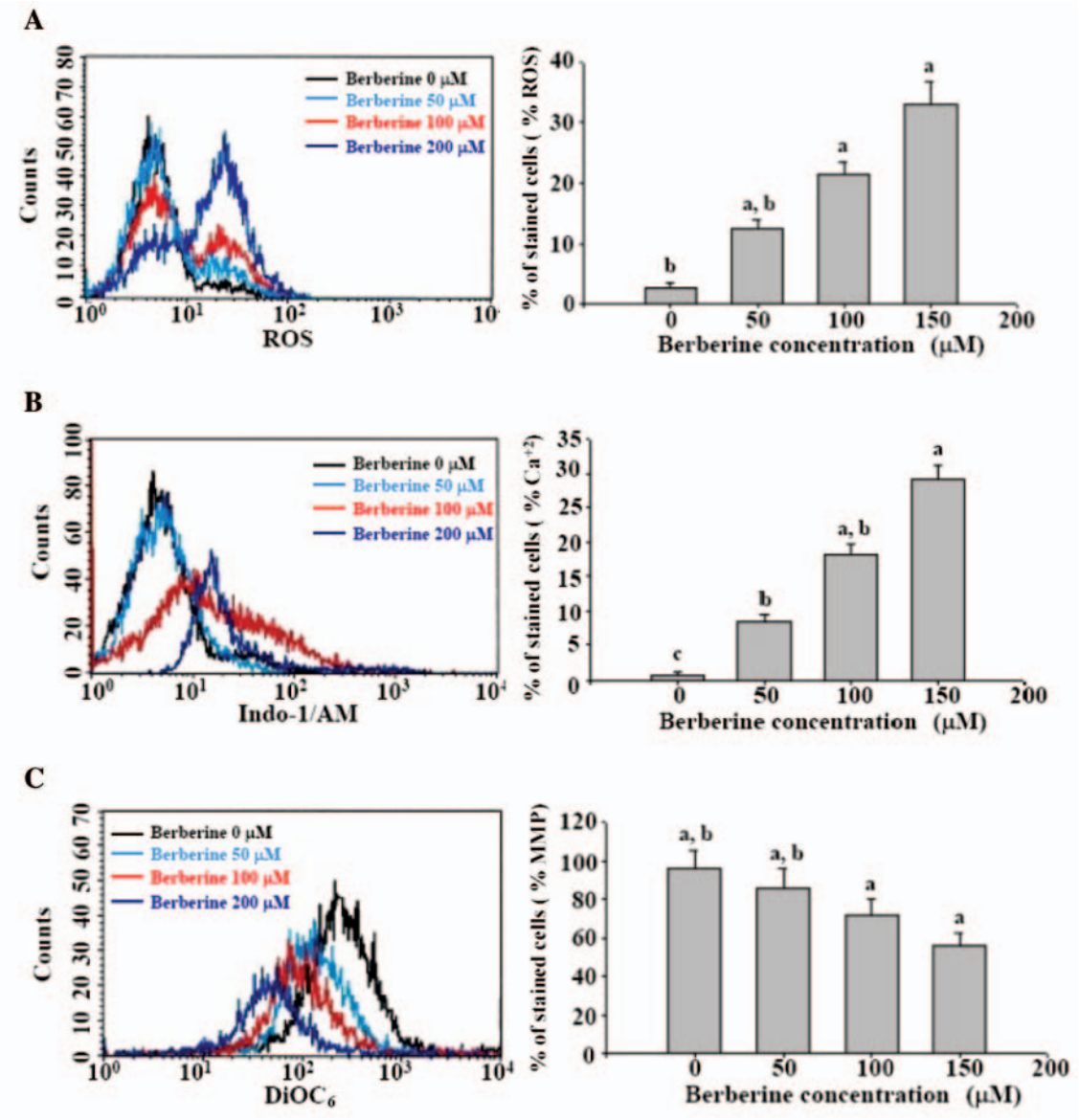

Figure 3. *Flow cytometric analysis of reactive oxygen species (ROS), Ca^{2+} and $\Delta\Psi_m$ in human CaSki cells treated with berberine for 24 h. The CaSki cells were treated with 0, 50, 100 and 150 μM berberine. Zero concentration was defined as control. The percentage of cells that were stained by DCFH-DA dye for ROS (A), by Indo-1/AM dye for Ca^{2+} (B) and by $DiOC_6$, dye for $\Delta\psi_m$ (C) were determined by flow cytometry as described in the Materials and Methods section. Values are means±SD (n=3).* [abc]*Groups not sharing the same letter were significantly different by Tukey's test ($p<0.05$).*

(Indo 1/AM) (3 μg/ml) and incubated at 37˚C for 30 min and the Ca^{2+} production was analyzed by flow cytometry as described previously (9,13-14).

Analysis of mitochondrial membrane potential ($\Delta\Psi_m$). Approximately, 2x10^5 cells /well of CaSki cells in 12-well plates with 0, 50, 100 and 150 μM berberine were incubated for 24 h. The cells were harvested and washed twice, re-suspended in 500 ml of 3,3'-dihexyloxcarbocyanine ($DiOC_6$) (4 mol/l) and incubated at 37˚C for 30 min. The $\Delta\Psi_m$ was analyzed by flow cytometry, as described previously (13).

Western blotting to examinine the effect of berberine on GADD153, p53, Bax, Bcl-2, cytochrome c and caspase-3 of CaSki cells. Approximately 2x10^6 cells/well of CaSki cells in 6-well plates with 0, 50, 100 and 150 μM berberine were incubated for 24 h. The total proteins were collected from the CaSki cells after treatment with berberine to xamine GADD153, p53, Bax, Bcl-2, cytochrome *c* and caspase-3 by using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously (9,13-14). A similar number of cells were pretreated with catalase (250 U/mL) followed by 100 μM berberine treatment before the levels of GADD153 were evaluated.

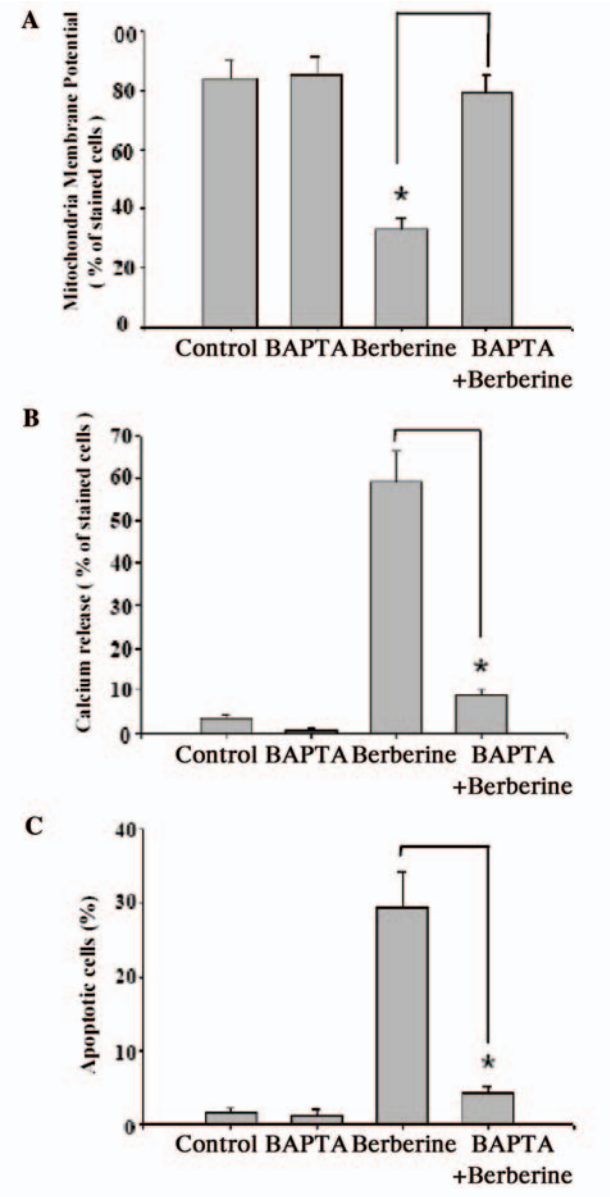

Figure 4. *Effects of calcium antagonist BAPTA (Ca^{2+} chelator) on berberine-induced changes in $\Delta\psi_m$, Ca^{2+} and apoptosis in Ca Ski cells. The Ca Ski cells were pre-treated with BAPTA for 3 h then treated with 100 μM berberine before analysis of $\Delta\psi_m$ (A), Ca^{2+} (B) and apoptosis (C). Data represent mean±S.D. of three experiments. Significantly different between berberine and BAPTA with berberine at *p<0.05.*

Statistical analysis. The Student's *t*-test and Tukey's test were used to analyze the statistical differences between the berberine-treated and control groups.

Results

Viable cells. Increasing the dose of berberine and/or the time of incubation led to a decrease in the percentage of viable cells as measured by flow cytometry. The effects of berberine were dose- and time-dependent (Figure 1A and B). The percentages of apoptosis after the CaSki cells were treated with 100 μM berberine for various time periods are shown in Figure 1C and D. Berberine induced apoptosis in a time-dependent manner.

Caspase-3 activation. Firstly, the capase-3 activity was determined by using flow cytometric analysis and the results indicated that berberine induced caspase-3 activity in a dose-dependent manner (Figure 2A). When the CaSki cells were pretreated with z-VAD-fmk and then with berberine, the results demonstrated that z-VAD-fmk inhibited berberine- induced caspase-3 activity which was accompanied by the marked attenuation of the berberine-induced apoptotic cell death (Figure 2B and C).

ROS, Ca^{2+} and $\Delta\Psi_m$ evaluations. The percentage of CaSki cells stained for ROS (Figure 3A), Ca^{2+} (Figure 3B) and $\Delta\Psi_m$ (Figure 3C) was significantly different between the berberine-treated groups and the controls. These effects were dose-dependent (Figure 3A). In the CaSki cells that were pretreated with BAPTA lower levels of Ca^{2+} release (Figure 4A), MMP disruption (Figure 4B) and percentage of apoptotic cells (Figure 4C) were found. Apparently, Ca^{2+} plays an important role in berberine-induced apoptosis in CaSki cells.

Western blot analysis. The results (Figure 5 A: GADD153, p53, Bax, Bcl-2 and cytochrome *c*; B: caspase-3 and C: cytochrome *c* in mitochondria and cytosol) from Western blot indicated that the levels of GADD153, Bax, cytochrome *c* and caspase-3 increased and Bcl-2 decreased. The results (Figure 5C) also indicated that berberine promoted cytochrome *c* release from the mitochondria. Additionally, GADD153 expression was abrogated when the CaSki cells were pretreated with catalase before berberine treatment (Figure 5D).

Discussion

Berberine decreased the percentage of viable cells and induced apoptosis in the CaSki cells in a dose-and time-dependent manner (Figures 1 and 2). This is in agreement with other reports which demonstrated that berberine induced apoptosis in human leukemia HL-60 cells (13), Ehrlich ascites carcinoma cells (15) and human gastric carcinoma SNU-5 cells (12). These results suggest that berberine may be an effective chemotherapeutic agent against cervical cancer. Our previous studies have demonstrated that berberine induced G1 arrest of the cell cycle in HL-60 cells and in the present study we also found out that berberine induced G1 arrest in the CaSki cells (data not shown). It is well known that the growth arrest of cancer cells in the G1-, S- and/or G2-phase provides an opportunity for cells to either undergo repair or apoptosis.

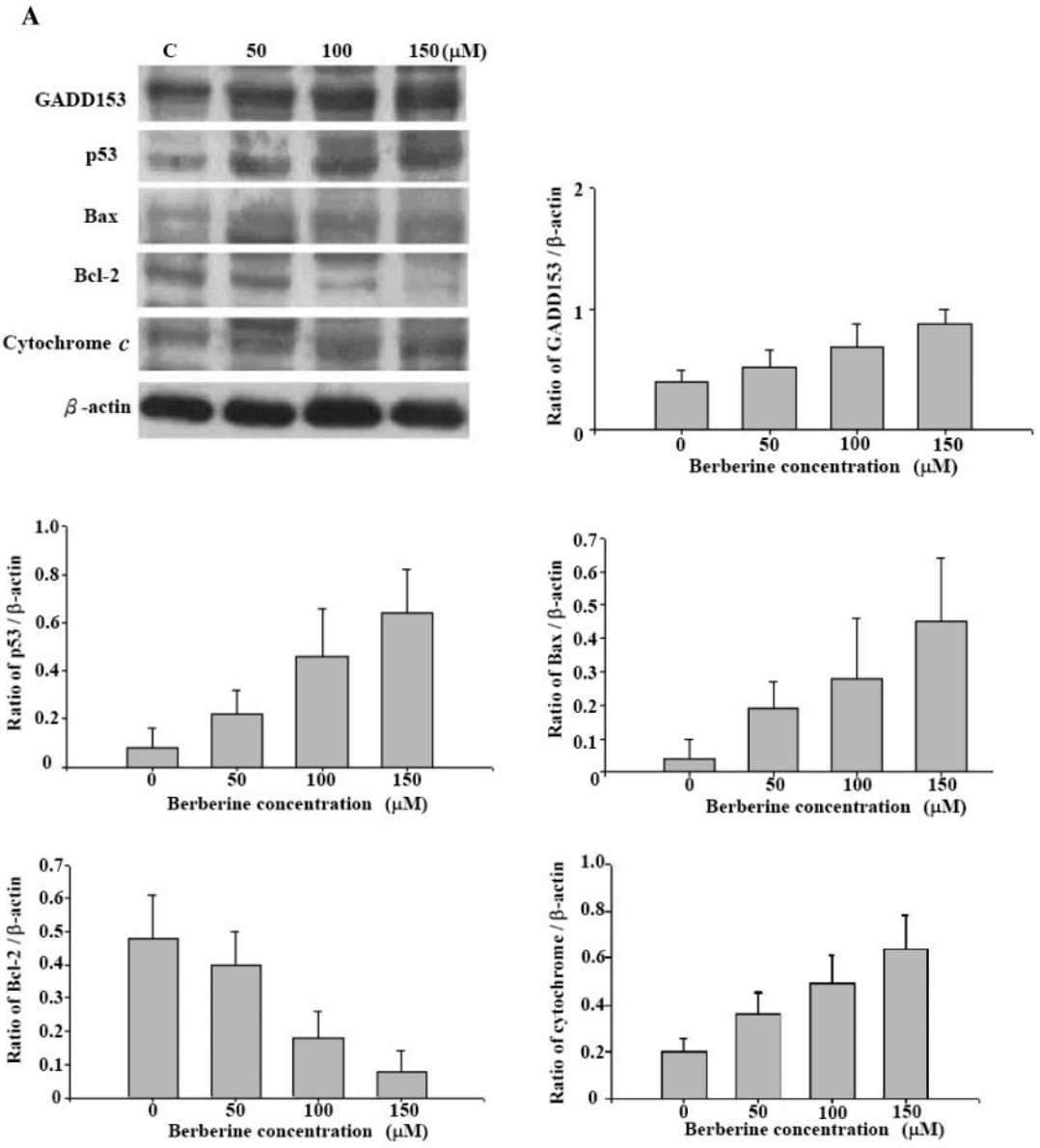

Figure 5. *continued*

Apoptosis has been considered as a protective mechanism against the development of cancer (16) and acquired resistance towards apoptosis can act as a hallmark of most carcinomas.

Berberine promoted the levels of ROS and Ca^{2+} but decreased $\Delta\Psi_m$ in the CaSki cells (Figure 3). This is in agreement with our previous reports in HL-60 cells (13) and it has also been reported that berberine induced disruption of mitochondria and induced apoptosis through caspase-3 activation in human epidermoid carcinoma A431 cells (16). However there is no information available regarding berberine-induced GADD153 levels in cells undergoing apoptosis. It has been reported that GADD153 is ubiquitously expressed at very low levels in growing cells, but it is highly expressed in response to numerous cellular stresses (22). Agents strongly affecting ER function have been identified as strong inducers of GADD153, including thapsigargin which depletes ER calcium stores, tunicamycin which blocks protein glycosylation, and dithiothreitol which disrupts disulfide bond formation (18). It is suggested that induction of GADD153 is highly responsive to ER stress. Our results clearly demonstrated that berberine induced increased levels of GADD153 and this was associated with the release of Ca^{2+} and with decreased $\Delta\Psi_m$ and was

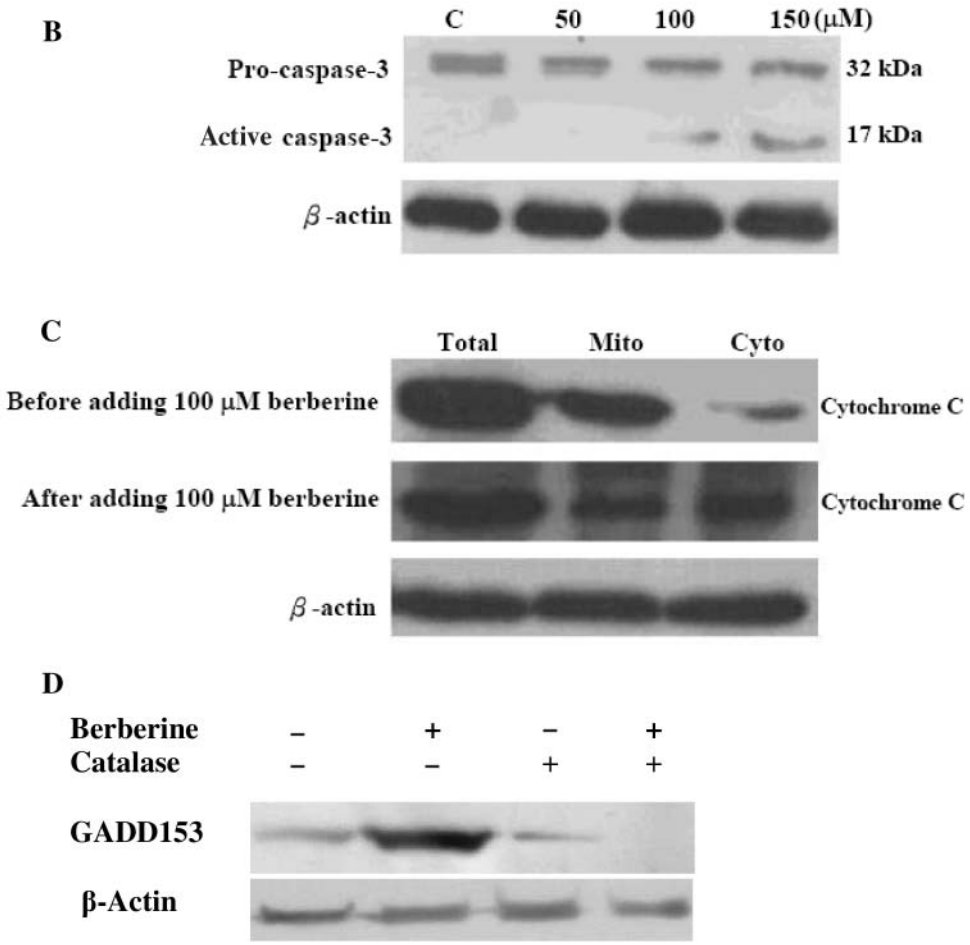

Figure 5. *Effects of berberine on the levels of GADD153, p53, Bax, Bcl-2, cytochrome c and caspase-3 from CaSki cells. The CaSki cells were treated with 0, 50, 100 or 150 μM berberine for 48 h. The evaluation of A: GADD153, p53, Bax, Bcl-2, cytochrome c; B: caspase-3; C: total: cytosolic and mitochondrial cytochrome c, and D: GADD153 after catalase preatment were estimated by Western blotting.*

positively associated with apoptosis. When the CaSki cells were pretreated with catalase before treatment with berberine, the GADD153 levels decreased (Figure 6). Pretreatment with BAPTA (a chelator of Ca^{2+}) before berberine treatment led to a decrease of apoptosis and caspase-3 activation in the CaSki cells.

It is well known that the ER is a predominant site for protein synthesis and folding, and for cellular calcium storage (19). It has also been reported that the accumulation of misfolded proteins and changes in Ca^{2+} homeostasis in the ER result in ER stress and lead to apoptotic cell death (20). Our data also showed that berberine induced increased ROS levels in CaSki cells. Berberine treatment increased the levels of p53, Bax, cytochrome *c*, caspase-3 and decreased the levels of Bcl-2. The proteins of the Bcl-2 family either promote cell survival (Bcl-2 and Bcl-xL) or induce apoptosis (Bax) (21-24). The changes of both levels of Bax and/or Bcl-2 leads to the loss of mitochondrial membrane potential which is a key event in the induction of apoptosis, and involves a reduction in ATP levels, influx of ions that leads to a decrease in mitochondrial membrane potential and the opening of mitochondrial permeability transition pores (24).

In conclusion, we suggest berberine induced ROS production followed by increasing GADD153 expression before leading to a change in $\Delta\Psi_m$ and dysfunction of the mitochondria followed by cytochrome c release and caspase-3 activation, finally leading to apoptosis (Figure 6). It is also suggested that ER can act as a third subcellular compartment implicated in apoptotic execution.

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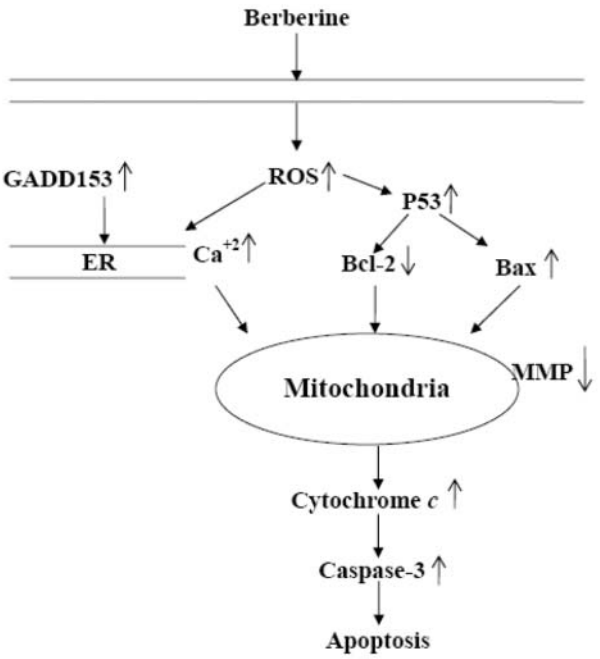

Figure 6. *Proposed mechanism of action for berberine-induced apoptosis in Ca Ski cells. Berberine induced ER stress, GADD153 expression then led to Ca^{2+} release, decreased the levels of $\Delta\psi_m$, leading to caspase-3 activity before causing apoptosis in CaSki cells.*

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