The Role of Ca^{+2} on Rhein-induced Apoptosis in Human Cervical Cancer Ca Ski Cells

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Abstract. *Apoptosis induced by rhein, an active component of senna, has been reported in various human cancer cells, however, its molecular mechanisms are not precisely known. In this study, the mechanisms of apoptosis by which rhein acts on human cervical cancer Ca Ski cells were examined. Flow cytometric analysis demonstrated that rhein induced the abrogation of mitochondrial membrane potential (MMP) and cleavage of Bid protein. Rhein also induced an increase in the levels of Fas, p53, p21 and Bax, but a decrease in the level of Bcl-2. The activities of both caspase-8 and -9 were enhanced by rhein, promoting caspase-3 activation, leading to DNA fragmentation, thus, indicating that rhein-induced apoptosis is caspase-dependent. In addition, rhein induced an increase in the level of cytoplasmic Ca^{2+}, which was inhibited by BAPTA (a calcium chelator). BAPTA attenuated the MMP abrogation and significantly dinimished the occurrence of rhein-induced apoptosis in Ca Ski cells. In conclusion, our data demonstrate that rhein-induced apoptosis occurs via a caspase-dependent and mitochondria-dependent pathway which is closely related to the level of cytoplasmic Ca^{2+} in Ca Ski cells.*

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), an anthraquinone compound in the root of rhubarb (*R. palmatum L*. or *R. tanguticum Maxim*), has been extensively used to treat chronic liver disease for a long time in China. Rhein inhibited the growth of tumor cells in rat liver (1), human glioma (2), and Ehrlich ascites tumor (3) *in vivo*. Rhein induced apoptosis in human colonic adenocarcinoma monolayer cells through generating nitric oxide *in vitro* (4) and induced apoptosis *via* a ROS-independent mitochondrial death pathway in human promyelocytic leukemia cells (HL-60) (5). Rhein inhibited human hepatoblastoma G2 cell growth *via* cell cycle arrest and apoptosis, related to p53 and the CD95/CD95L apoptotic system (6). Rhein blocked IL-12 mRNA transcription through lowering $[Ca^{2+}]$ and inhibition of protein kinase C (7). In addition, rhein was found to inhibit superoxide anion production, chemotaxis and phagocytic activity of neutrophils, and macrophage migration and phagocytosis (8).

Apoptosis is a well regulated and organized death process that occurs under a variety of physiological and pathological conditions that control the development and homeostasis of multicellular organisms (9). The apoptotic features comprise cellular morphological change, membrane blebbing, chromatin condensation, oligonucleosomal DNA cleavage, translocation of phosphatidylserine of the plasma membrane from the inner to the outer leaflet, and activation of a family of caspases (10-12). Caspase activation is generally considered to be a key hallmark of apoptosis. Although rhein has been demonstrated to induce apoptosis in HL-60 cells through the ROS-independent mitochondrial death pathway, no information for rhein effect on human cervical cancer Ca Ski cells is available. Therefore, in this study we focused on the molecular mechanism and the role of Ca^{2+} on the induction of apoptosis induced by rhein in Ca Ski cells.

Materials and Methods

Chemicals and reagents. Rhein, propidium iodide (PI), RNase, trypan blue, Tris-HCl and Triton X-100 were obtained from Sigma Chemical Co. BAPTA, potassium phosphates and dimethyl sulfoxide (DMSO) were obtained from Merck Co. (Darmstadt, Germany). RPMI-1640, penicillin-streptomycin, trypsin-EDTA,

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fetal bovine serum (FBS) and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kit was bought from OncoImmunin, Inc. (Gaithersburg, MD, USA).

Human cervical epidermoid carcinoma cell line (Ca Ski). Ca Ski cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Ca Ski cells were cultured for several generations and the viability of each generation was checked. 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 Atm in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% L-glutamine.

Morphological changes and cell viability of Ca Ski cells treated with or without rhein were examined and determined using Phase-light microscopy and flow cytometry. The Ca Ski cells were plated in 12-well plates at a density of $2x10^5$ cells/well and grown for 24 h. Different concentrations of rhein were then added to cells for a final concentration of 0, 5, 10, 25, 50 and 100 μM, while DMSO (solvent) alone was added to the control group. The cells were grown at 37˚C, 5% CO_2 and 95% air for different periods of time. Morphological changes of cells were observed and photographed using phase-contrast light microscopy. In order to determine cell viability, PI stain was used and a flow cytometric assay was carried out as described elsewhere (13, 14).

DAPI staining for the effects of rhein on apoptosis in Ca Ski cells. Approximately $2x10^5$ cells/well of Ca Ski cells were grown in 6-well plates and treated with various concentrations of rhein for 48 h prior to staining. Cells were washed three times with PBS and fixed with 4% paraformaldehyde. Fixed cells were washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml, Sigma) for 30 min. Stained cells were then examined under fluorescent microscopy photographed and apoptotic cells identified (15).

DNA laddering fragmentation. Approximately $1x10^6$ cells/well of Ca Ski cells were grown in 6-well plates and treated with rhein at 50 μM for 24, 48 and 72 h. DNA from treated and non-treated rhein cells was isolated, examined in 0.8% agarose gel electrophoresis and photographed under fluorescence microscopy as described elsewhere (13).

Caspase-3 activity determination of Ca Ski cells treated with or without rhein. Approximately $2x10^5$ cells/well of Ca Ski cells in 12-well plates of concentrations of 0, 5, 10, 25, 50 and 100 μM of rhein were incubated in an incubator for different time periods. Cells were harvested by centrifugation and the medium was removed before adding 50 μL of 10 μM substrate solution to the cell pellet ($1x10^5$ cells per sample). We did not vortex the cells. Cells were incubated at 37˚C for 60 min then washed once with 1 mL of ice-cold PBS and re-suspended in 1 mL fresh PBS. Cells were analyzed with a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at 488 nm. Caspase-3 activity was determined and analyzed (16, 17) according to manufactories instructions.

Detection of Reactive oxygen species (ROS) in Ca Ski cells after treatment with rhein. The level of ROS of the Ca Ski cells was examined with flow cytometry (FACS Calibur, Becton Dickinson), using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). Approximately $2x10^5$ cells/well of Ca Ski cells in 12-well plates were treated with 50 μM rhein for 6, 12, 24, 48 and 72 h. The cells were harvested and washed twice, re-suspended in 500 μl of DCFH-DA (10 μM) and incubated at 37˚C for 30 min, then analyzed to detect the changes of ROS by flow cytometry (17).

Detection of Mitochondrial membrane potential (MMP) in Ca Ski cells after treatment with rhein. The mitochondrial membrane potential of the Ca Ski cells was determined with flow cytometry using $DiOC_6$ (4 mol/L). Approximately $2x10^5$ cells/well of Ca Ski cells in 12-well plates were treated with 50 μM rhein for 0.5, 1, 2, 4 and 6 h. The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 mol/L) and incubated at 37˚C for 30 min before being analyzed to detect the changes of mitochondrial membrane potential (MMP) using flow cytometry (17).

Detection of the levels of cytoplasmic Ca^{2+} in rhein-treated Ca Ski cells using Flow cytometry. The level of cytoplasmic Ca^{2+} of the Ca Ski cells was determined using Indo 1/AM (Calbiochem, La Jolla, CA, USA) by flow cytometry. Approximately $2x10^5$ cells/well of Ca Ski cells in 12-well plates were treated with 50 μM rhein for 1, 3, 6, 12 and 24 h. The cells were harvested and washed twice, re-suspended in Indo 1/AM (3 μg/ml), incubated at 37˚C for 30 min, then analyzed to detect the changes of cytoplasmic Ca^{2+} levels using flow cytometry (17, 18).

Detection of the levels of cytoplasmic Ca^{2+} and MMP in Ca Ski cells pre-treated with BAPTA then treated with rhein. Approximately $2x10^5$ cells/well of Ca Ski cells in 12-well plates were pre-treated with BAPTA before the addition of 50 μM rhein for 24 hours incubation. The cells were harvested and washed twice, once for apoptosis analysis and the other for re-suspension in Indo 1/AM (3 μg/ml) or $DiOC_6$ (4 mol/L) before being incubated at 37˚C for 30 min, then analyzed by flow cytometry. As described above, Indo 1/AM was used for the detection of Ca^{2+} and $DiOC_6$ (4 mol/L) for the detection of MMP. The levels of cytoplasmic Ca^{2+} and MMP of the Ca Ski cells were then determined.

Western blotting to examine the effect of rhein on p53, Bax, Bcl-2, caspase-3,-8, and -9,Bid and cytochrome c of Ca Ski cells. The total proteins were collected from Ca Ski cells treated with or without 50 μM of rhein for 6, 12, 24, and 48 h, and p53, Bax, Bcl-2, caspase-3, -8, and -9, Bid and cytochrome *c* expression were examined using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as described previously (13, 14).

Statistical analysis. Student's *t*-test was used to analyze the differences between the rhein treated and control groups.

Results

Effects of rhein on morphological changes and cell viability of human cervical cancer Ca Ski cells. Morphological changes induced by rhein on Ca Ski cells revealed both necrotic and apoptotic cell death. A proportion of the cells revealed swelling, cell membrane lysis and disintegration of organelles indicating necrosis, and the other cells revealed shrinkage and nuclear condensation suggesting apoptosis of the cells under fluorescent microscopy (Figure 1A). Using flow cytometry, the effect of rhein on the viability of Ca Ski cells revealed a dose-dependent relationship (Figure 1B).

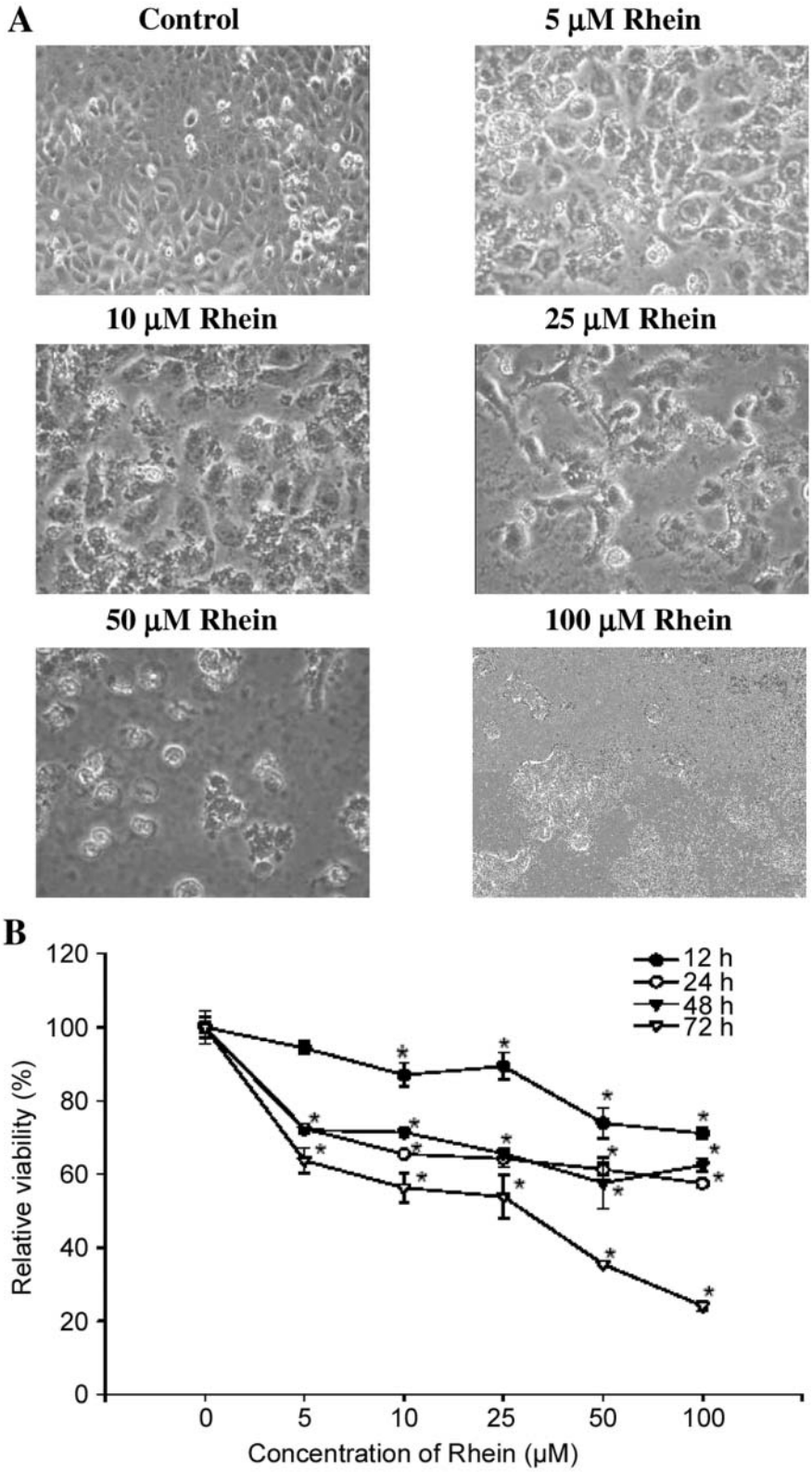


Figure 1. *The morphological changes and the percentage of viable Ca Ski cells after rhein treatment. The Ca Ski cells (2x10^5 cells/well; 12-well plates) were plated in RPMI-1640 +10% FBS with different concentrations of rhein for 48 h. The morphological changes of Ca Ski cells were photographed under phase-light microscopy (A). The cells were collected by centrifugation and the viable cells were determined using trypan blue exclusion, propidium iodine and flow cytometry, as described in the "Materials and Methods" (B). *p<0.05*

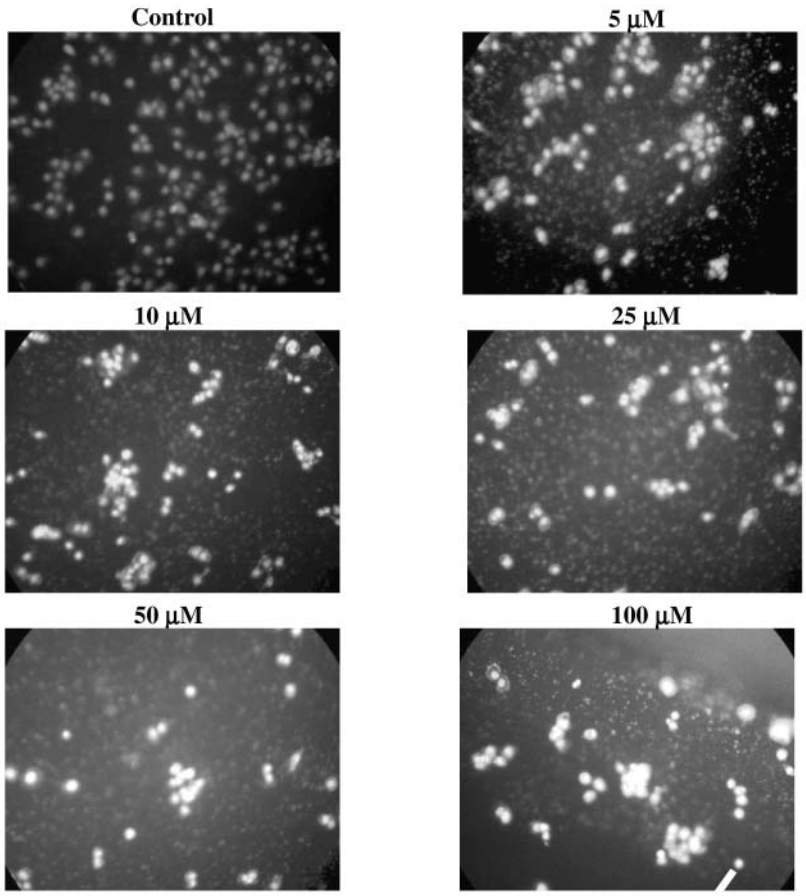


Figure 2. *DAPI staining analysis for the effects of rhein on Ca Ski cell apoptosis. The Ca Ski cells were incubated with various concentrations of rhein for 48 h and apoptosis was determined using DAPI staining, as described in the "Materials and Methods".*

Induction of apoptosis by rhein on Ca Ski cells using DAPI staining. We studied the occurrence of apoptosis of Ca Ski cells brought about by rhein treatment. Apoptosis was detected using DAPI staining after 48 h of continuous exposure to rhein as determined using fluorescence microscopy. As shown in Figure 2, rhein induced apoptosis in a concentration-dependent manner.

Induction of DNA fragmentation by rhein on Ca Ski cells. We studied the occurrence of DNA fragmentation from Ca Ski cells treated with rhein at 50 μM using DNA gel electrophoresis. As shown in Figure 3, rhein induced DNA fragmentation in a concentration-dependent manner.

Effects of rhein on caspase-3 activity in Ca Ski cells. Using flow cytometry we demonstrated that caspase-3 activity was increased in the rhein-treated group compared to that of the control group. The increase of caspase-3 activity in Ca Ski cells treated with 50 μM rhein was enhanced from 6 to 72 hours (Figure 4).

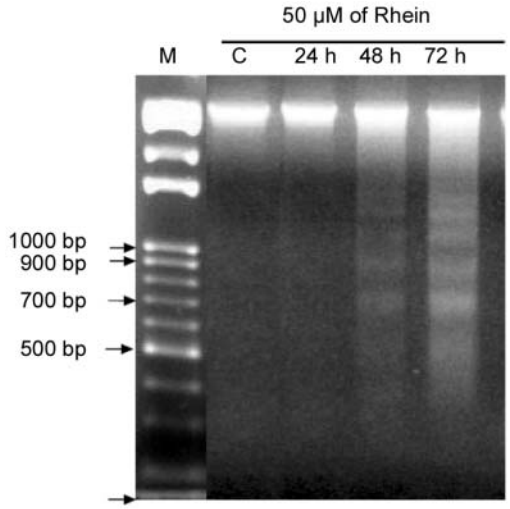


Figure 3. *DNA fragmentation determination for the apoptotic effects of rhein on Ca Ski cell. The Ca Ski cells were incubated with 50 μM rhein for 24, 48 and 72 h, and DNA fragmentation was determined using DNA gel electrophoresis, as described in the "Materials and Methods".*

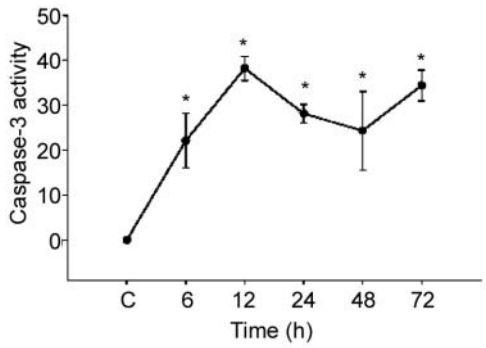


Figure 4. *Flow cytometric assays of the effects of rhein on caspase-3 activity in Ca Ski cell. The Ca Ski cells were incubated with 50 μM rhein for 6, 12, 24, 48 and 72 h before cells were harvested for caspase-3 activity determination as described in the "Materials and Methods". Data represent mean±S.D. of three experiments. *p<0.05.*

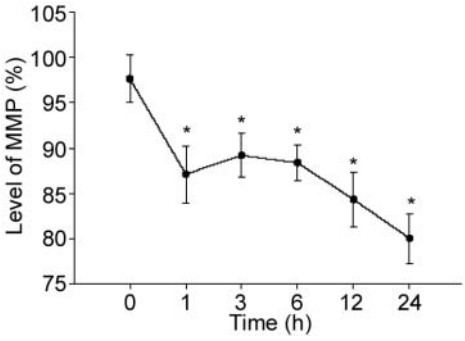


Figure 6. *Flow cytometric analysis of mitochondrial membrane potential (MMP) in human Ca Ski cells (5x10^5 cells/ml) treated with 50 μM rhein for 0, 1, 3, 6, and 12 h. The zero concentration was defined as the control. Levels of MMP were detected using DiOC$_6$ dye determined as described in the "Materials and Methods" section. *Significant difference between rhein-treated cells and control. p<0.05.*

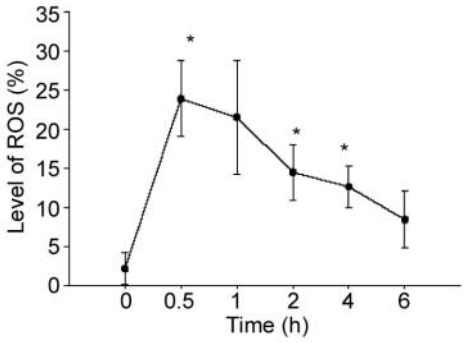


Figure 5. *Flow cytometric analysis of reactive oxygen species (ROS) in human Ca Ski cells (5x10^5 cells/ml) treated with 50 μM rhein for 0, 0.5, 1, 2, 4 and 6 h. The zero concentration was defined as the control. ROS were stained by DCFH-DA dye and determined as described in the "Materials and Methods" section. *Significant difference between the rhein-treated cells and the control, p<0.05.*

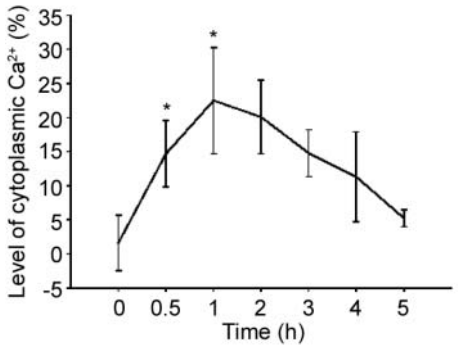


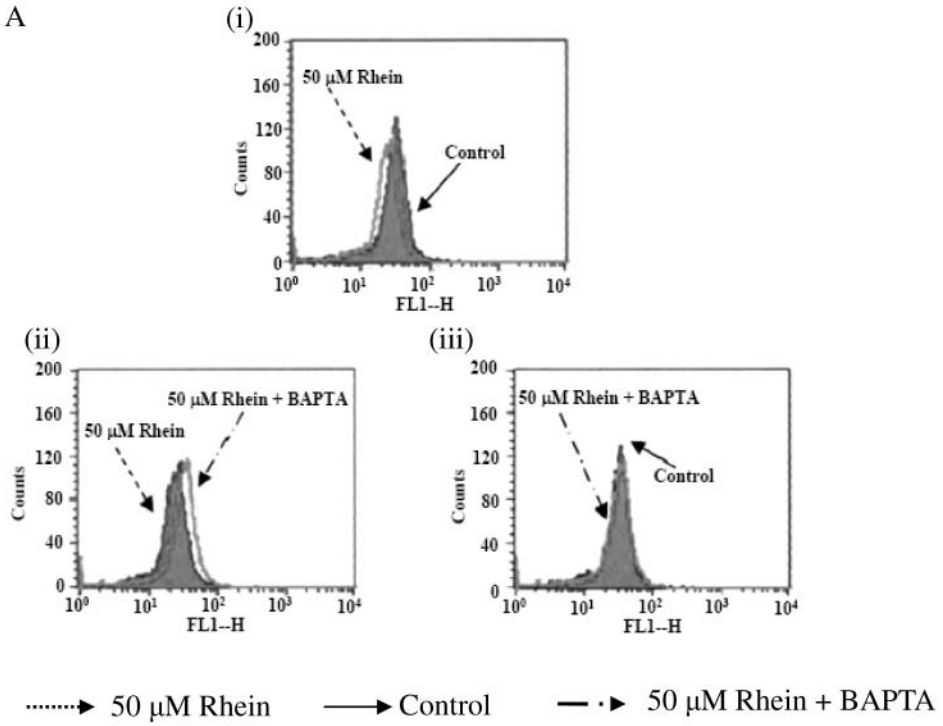
Figure 7. *Flow cytometric analysis of cytoplasmic Ca^{2+} levels in human Ca Ski cells (5x10^5 cells/ml) with 50 μM rhein for 0, 0.5, 1, 2, 3, 4 and 6 h. The zero concentration was defined as the control. On exposure to rhein, the cytoplasmic Ca^{2+} increased approximately to 20% in 1 h, and then returned to control level in 6 h in examined cells. *Significant difference between rhein-treated cells and control. p<0.05.*

Detection of reactive oxygen species (ROS) in Ca Ski cells after treatment with rhein. Levels of ROS increased in the rhein-treated group compared to the control group. The ROS level was found to reach a peak at 30 min, and subsequently decreased in Ca Ski cells exposed to rhein at 50 μM. Levels of ROS remained higher in the rhein-treated group than in the control group for at least for 4 hours (Figure 5).

Effects of rhein on mitochondrial membrane potential of Ca Ski cells. Mitochondrial membrane potential (MMP) was lower in the rhein-treated group. The decrease of MMP in the examined Ca Ski cells was generally inversely proportional to the duration of rhein treatment from 1 to 24 hours at 50 μM, revealing a time-dependent effect (Figure 6).

Effects of rhein on cytoplasmic Ca^{2+} levels in Ca Ski cells. Cytoplasmic Ca^{2+} levels increased in the rhein-treated group than in the control group. On exposure to rhein at 50 μM, cytoplasmic Ca^{2+} increased to approximately 20% in one hour, and then returned to basal (control) level in 6 hours (Figure 7).

Effects of BAPTA on MMP and cytoplasmic Ca^{2+} in rhein-treated Ca Ski cells. Pre-treatment with BAPTA (a calcium chelator) greatly influenced the levels of MMP and cytoplasmic Ca^{2+} in rhein-treated Ca Ski cells (Figures 8 and 9). The decrease in MMP levels was inhibited by BAPTA pre-treatment in rhein-treated cells (Figure 8A and 8B). BAPTA also significantly suppressed the increase of cytoplasmic Ca^{2+} induced by rhein treatment (Figure 9A and 9B).



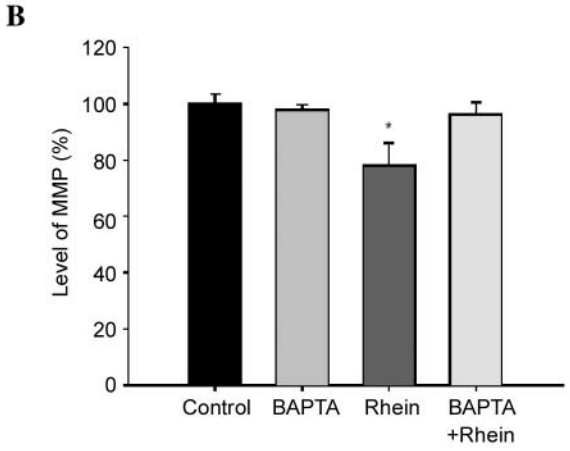
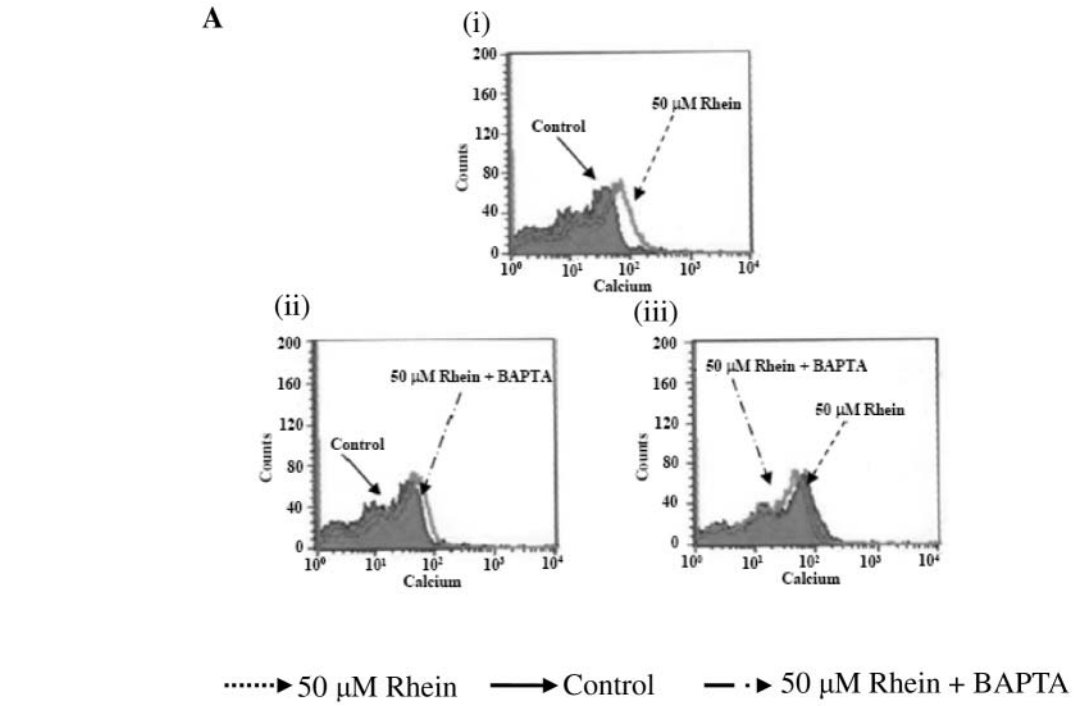


Figure 8. *Effects of the calcium chelator BAPTA on MMP of the rhein-treated Ca Ski cells. The Ca Ski cells were pre-treated with BAPTA for 3 h then treated with rhein before cells were harvested for MMP determination, as described in the "Materials and Methods". Data represent mean±S.D. of three experiments. *p<0.05. (A) Representative profiles and (B) MMP levels.*

Western blotting for the effects of rhein on p53, cytochrome c, caspases-3, -8 and -9, Fas, Bax, Bcl-2, and Bid in Ca Ski cells. The results of the Western blot are shown in Figure 10. The increase in the expression of p53, cytochrome *c*, Bax, tBid, caspases-3 and -9, and the decrease in the expression of Bcl-2 may contribute to the occurrence of apoptosis of the cells examined.

Discussion

Rhein is an anthraquinone compound existing in rhubarb (a traditional Chinese medicinal plant). It has been proven that inhibition of the growth of tumor cells may be associated with apoptotic cell death. In this study, we explored the mechanisms by which rhein, an active component of senna, acts on a human cervical cancer cell line (Ca Ski) to induce apoptosis through the mitochondria-dependent pathway. We showed that rhein decreased the percentage of viable cells in the Ca Ski cell line in a dose- and time-dependent manner. Rhein may induce cell death through either necrosis or apoptosis in Ca Ski cells. The IC_{50} of rhein for Ca Ski cells was 25 μM in our study; in comparison to other cell types, the IC_{50} of rhein for KB, hepatoma BEL-7402 and mammary carcinoma MCF-7 cells was 11.5 $\mu g/mL^{-1}$, 14.0 $\mu g/mL^{-1}$ and 18.4 $\mu g/mL^{-1}$, respectively (19). Apparently, the



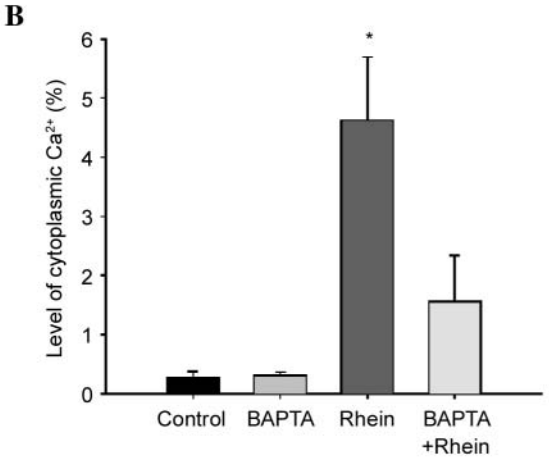


Figure 9. *Effects of the calcium chelator BAPTA on cytoplasmic Ca^{2+} levels in Ca Ski cells treated by rhein. The Ca Ski cells were pre-treated with BAPTA for 3 h then treated with rhein before cells were harvested for Ca^{2+} determination, as described in the "Materials and Methods". Data represent mean±S.D. of three experiments. *p<0.05. (A) Representative profiles and (B) MMP levels.*

IC_{50} of rhein differs between various cell lines, which indicates that the sensitivity of tumor cells to rhein is dependent on cell type. We then demonstrated that incubation of cells with various concentrations of rhein led to apoptosis triggering (sub-G_1 group), which appeared to be mediated by a caspase-dependent pathway in a dose- and time-dependent fashion. This is in agreement with other reports which showed that rhein induced apoptosis in human colonic adenocarcinoma monolayer cells (4) and human promyelocytic leukemia cells (HL-60) (5).

Based on reports regarding Chinese medicine, the root of rhubarb was used at an average daily dosage of 2-20 g of powered root for stomach cancer, leukemia and liver cancer (20, 21). The interesting point is that rhubarb root contains 3-12% anthraquinones which include 60-80% rhein, emodin, and aloe-emodin (equivalent to approximately 400 μM in the blood). Many studies have demonstrated that emodin and aloe-emodin induced cell cycle arrest and apoptosis in human and animal cancer cell lines, but there are no reports that show rhein affecting a human cervical cancer cell line. Our data demonstrated that 50 μM rhein induced apoptosis at 12, 24, 46 and 72 h in the examined Ca Ski cells. Thus, the concentration used in the present study is comparable to the daily therapeutic dosage of rhubarb root, which indicates

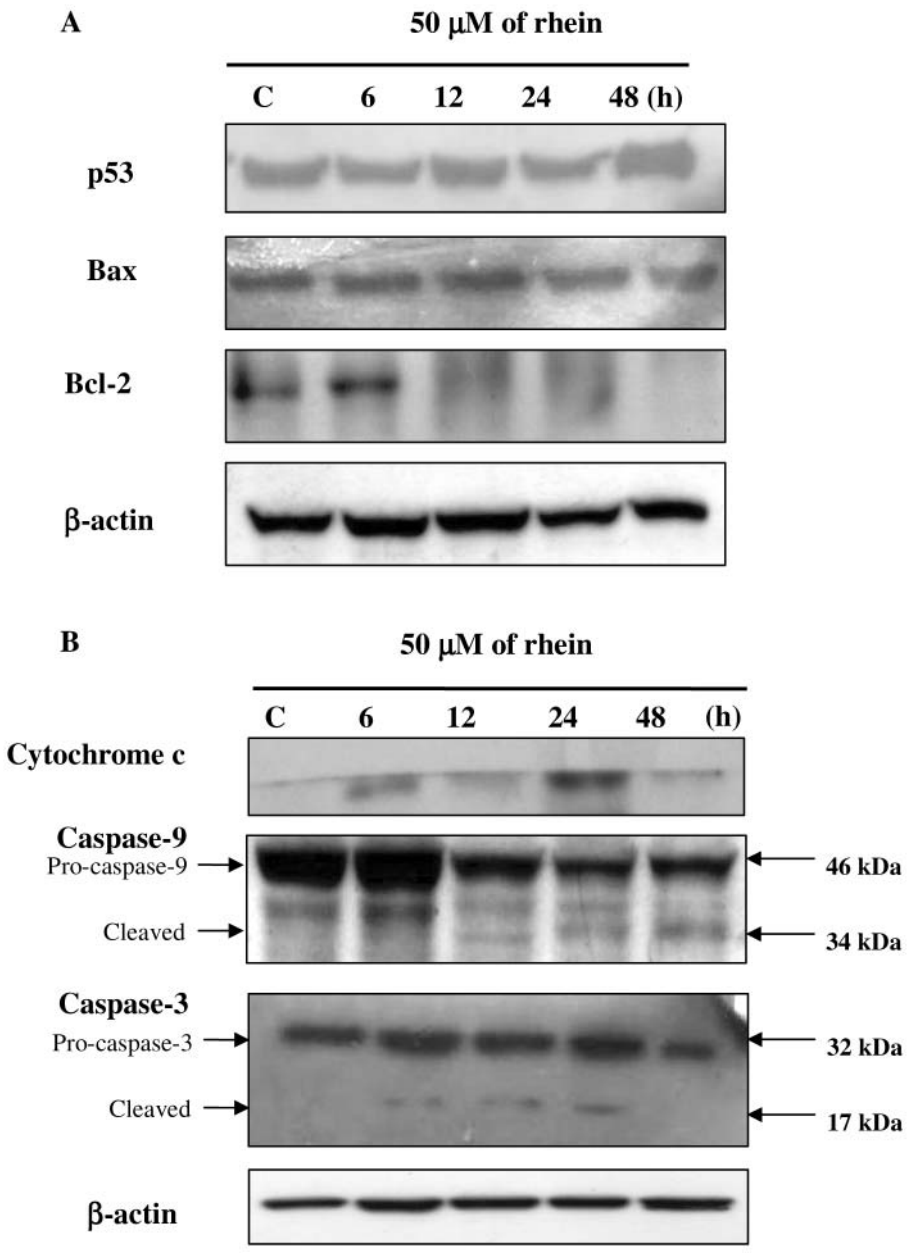
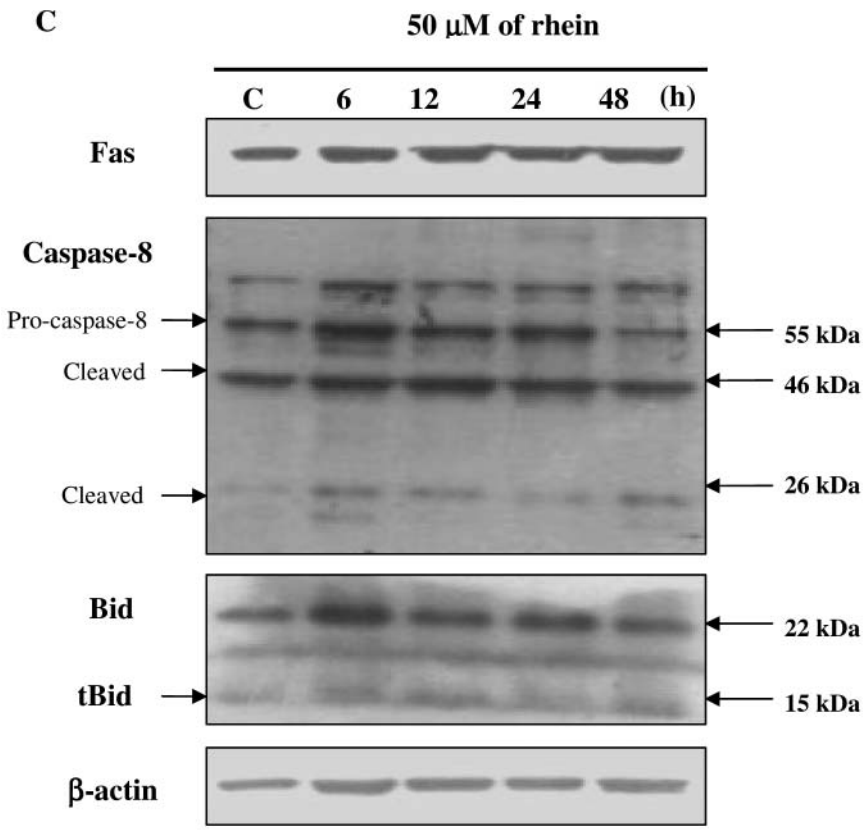


Figure 10. *Representative Western blot showing changes on the levels of p53, bax, Bcl-2, cytochrome c, caspase-9, -3, and -8, and Bid in Ca Ski cells ($5x10^6$/ml) after treatment with rhein (50 μM) for 6, 12, 24 and 48 h. (A) p53, Bax, Bcl-2; (B) cytochrome c, caspase-9 and -3; (C) Fas, caspase-8 and Bid. Expressions were estimated using Western blotting, as described in the "Materials and Methods".*

that long-term treatment with 50 μM rhein may cause apoptosis induction in tumor cells. The induction of apoptosis by rhein in an animal model *in vivo* for cervical cancer needs to be further investigated.

Rhein-induced apoptosis in Ca Ski cells was supported by the: (i) occurrence of sub-G_1 group by flow cytometry; (ii) demonstration of DAPI staining of cells under fluorescence microscopy; (iii) fragmentation of DNA in gel electrophoresis. In addition, morphological examination revealed cell shrinkage, chromatin condensation and loss of cell-to-cell contact in rhein-treated Ca Ski cells. It was reported that the loss of substrate adherence is an early event in apoptosis of colon adenoma cells after treatment with an apoptotic-inducing agent (22). The DNA damage caused by rhein in these studies were also confirmed by Comet assay that showed a dose-dependent effect.

Figure 10. *continued*



It has been proven that caspases play a critical role in the process of apoptosis, and two major apoptotic pathways, the caspase-dependent and caspase-independent, have been recognized. According to caspase substrate specificities and target proteins, caspases can be grouped into "apoptotic initiators", such as caspase-8, and "apoptotic effectors", such as caspase-3 (23). In this study, rhein-induced apoptosis was observed at 6 h to 72 h. We also showed rhein-induced cell cycle arrest at sub-G_1 group, DNA fragmentation, activation of caspase-8, -9 and -3 and promoted Bax expression, but decreased Bcl-2 expression in Ca Ski cells. We also used flow cytometry for caspase-3 activity determination and demonstrated that rhein promoted the activity of caspase-3, the primary executioner caspase for apoptosis. We also determined that adding the caspase-3 inhibitor, Ac-DEVD-CHO led to a decrease in apoptosis in Ca Ski cells treated with rhein (data not shown). These results demonstrated that rhein-induced apoptosis might involve a caspase-3-mediated mechanism and the activation of caspase-8 and -9 might act upstream of caspase-3 activation.

It has been documented that the mitochondrion plays a key role in the regulation of apoptosis (24, 25). Mitochondrial dysfunctions including loss of mitochondrial membrane potential (MMP), permeability transition, and release of cytochrome *c* from the mitochondrion into the cytosol are associated with apoptosis (26). Our data also showed that rhein induced a rapid loss of MMP and release of cytochrome *c* in Ca Ski cells. Caspase-8 can be activated either by cytokines or chemicals that in turn induce Bid cleavage, and cleaved Bid (tBid) causes the cefflux of cytochrome *c* from the mitochondrion, caspase-3 activation, and PARP cleavage, and finally results in apoptosis (27, 28). Our Western blot results showed that rhein decreased Bid, but increased tBid protein level in Ca Ski cells. Our data also showed that rhein-induced Fas and caspase-8 expressions may in turn lead to cleavage of Bid and hence mitochondrion-mediated cytochrome *c* release and sequential activation of caspase-9 and caspase-3. Thus, a mitochondria-dependent pathway is suggested to be involved in rhein-induced apoptosis in Ca Ski cells.

It has been reported that chemopreventive agents induce apoptosis in part with generation of ROS and the disruption of redox homeostasis (26, 29). Our data showed an induction of intracellular ROS levels by rhein observed using the DCHF-DA assay in Ca Ski cells; co-treatment with catalase, a scavenger of ROS, resulted in a decrease of ROS levels, but did not block rhein-induced apoptosis. The results of the present study suggest that apoptosis induced by rhein occurs through a mitochondria-dependent, ROS-independent pathway. Similar

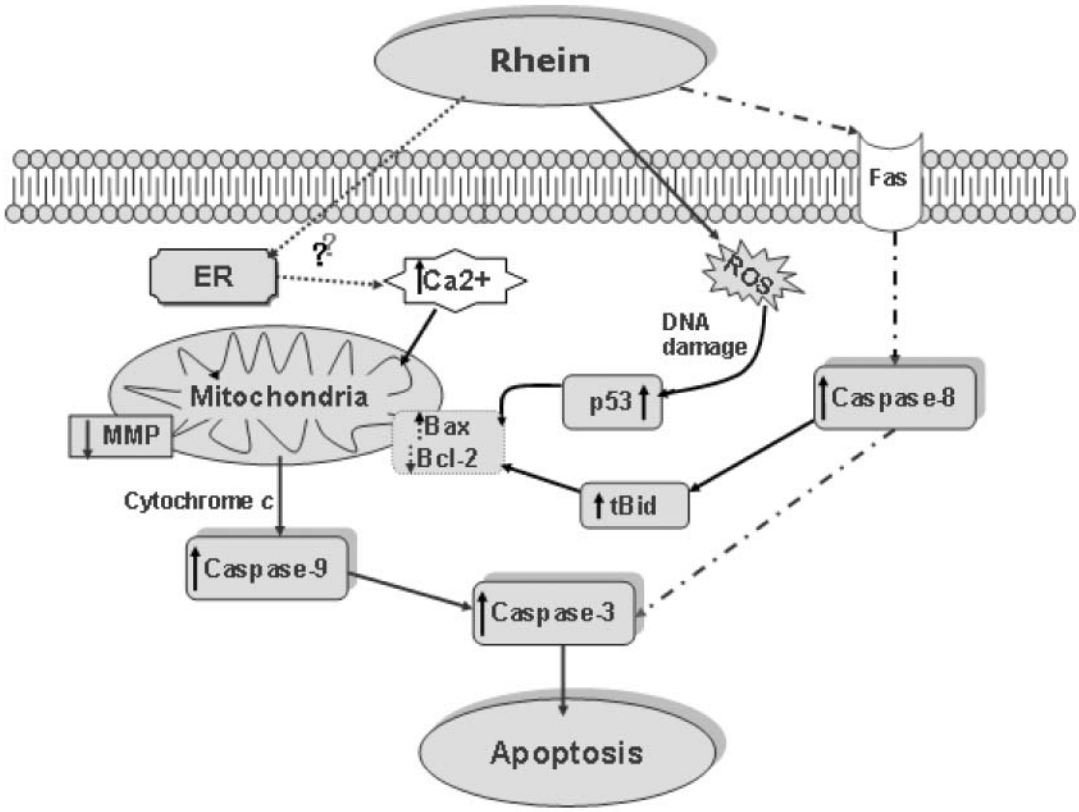


Figure 11. *Proposed model of rhein mechanism of action for apoptosis in Ca Ski cells. Rhein increased the production of ROS and Ca^{2+} production and decreased MMP levels leading to increase caspase-3 activity before causing apoptosis in Ca Ski cells.*

results were also reported in myricetin- and emodin-induced apoptosis in human leukemia HL-60 cells (5, 30).

We also found that rhein induced a swift increase in cytoplasmic Ca^{2+}, and pre-treatment with BAPTA led to a block of the increase of cytoplasmic Ca^{2+}, and a protection of against MMP reduction in Ca Ski cells. Furthermore, we also found that BAPTA blocked rhein-induced apoptosis in examined cells (data not shown). The increase of cytoplasmic Ca^{+2} is usually ascribed to endoplasmic reticulum (ER) stress, which results in Ca^{+2} release from the ER to the cytosol (31). Subsequently, cellular Ca^{2+} overload promotes mitochondrial Ca^{2+} uptake, subsequently contributing to mitochondrial permeability transition and release of mitochondrial apoptogenic factors into the cytosol (31). Thus, we suggest that Ca^{2+} plays a critical role in rhein-induced mitochondria-dependent apoptosis in Ca Ski cells.

In summary, this is the first report for rhein-induced apoptosis in human Ca Ski cervical cancer cells. Rhein-induced apoptosis is a caspase-3 and mitochondria-dependent pathway which also involves Ca^{2+} levels in these cells (Figure 11). These findings provide information about the therapeutic function of rhein in human cervical cancer.

Acknowledgements

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