

RESEARCH ARTICLE

Ursolic Acid Promotes Apoptosis of SGC-7901 Gastric Cancer Cells through ROCK/PTEN Mediated Mitochondrial Translocation of Cofilin-1

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Abstract

Ursolic acid, extracted from the traditional Chinese medicine bearberry, can induce apoptosis of gastric cancer cells. However, its pro-apoptotic mechanism still needs further investigation. More and more evidence demonstrates that mitochondrial translocation of cofilin-1 appears necessary for the regulation of apoptosis. Here, we report that ursolic acid (UA) potently induces the apoptosis of gastric cancer SGC-7901 cells. Further mechanistic studies revealed that the ROCK1/PTEN signaling pathway plays a critical role in UA-mediated mitochondrial translocation of cofilin-1 and apoptosis. These findings imply that induction of apoptosis by ursolic acid stems primarily from the activation of ROCK1 and PTEN, resulting in the translocation of cofilin-1 from cytoplasm to mitochondria, release of cytochrome c, activation of caspase-3 and caspase-9, and finally inducing apoptosis of gastric cancer SGC-7901 cells.

Keywords: Ursolic acid - apoptosis - ROCK/PTEN - cofilin-1 - mitochondrial translocation - gastric cancer

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Introduction

Ursolic acid (UA), extracted from bearberry, has been found a variety of effect in various tumours, such as cytotoxicity (Ma et al., 2005), growth inhibition (Wang et al., 2011), pro-apoptosis (Anderson et al., 2003). Our preliminary study has also found the pro-apoptotic effect of ursolic acid on gastric cancer cells SGC-7901, but its detailed mechanism still remains unclear.

Mitochondria are the major organelles involved in the process of promoting apoptosis (Wang, 2001). Some pro-apoptotic proteins could target mitochondria, change the permeability of the mitochondrial membrane, and make some apoptotic effectors like cytochrome c leak out (Green et al., 2004). Cytochrome c could release from mitochondria into the cytoplasm, activate caspase-9 by forming a complex with Apaf-1, and finally lead to the activation of caspase-3 (Li et al., 1997). Although the role of mitochondria in controlling downstream apoptotic events such as caspases activation is relatively well characterized, mechanisms by which upstream apoptotic signals remain largely elusive. It was reported that the activity of actin regulatory protein such as ADF/cofilin plays a critical role in the regulation of apoptosis in various mammalian cells (Bamburg et al., 2010), and the translocation of cofilin-1 from cytoplasm to mitochondria appears necessary for the opening of the mitochondrial permeability transition pore and subsequent release of cytochrome c into cytoplasm, activation of caspase-3,

caspase-9, which finally leads the initiation of apoptosis (Chua et al., 2003).

In our study, we provided the evidence firstly that, ursolic acid could induce the apoptosis of SGC-7901 cells through ROCK1/PTEN mediated transduction of cofilin-1 from cytoplasm to mitochondria, release of cytochrome c, and activation of caspase-3, caspase-9.

Materials and Methods

Chemicals and antibodies

Ursolic acid was purchased from Sigma (USA, order number: 89797-5MG-F, purity $\geq$ 98.5%). Y-27632 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cytochrome c, cofilin-1, ROCK1, PTEN and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA); cleaved caspase-3, cleaved caspase-9, and COXIV were purchased from Cell Signaling Technology (USA).

Cell culture

Human gastric cancer cells SGC-7901 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 g/ml streptomycin, 100 U/ml penicillin, 37°C, 5% CO₂, and high humidity.

Analysis of cells viability

Cell Counting Kit-8 (CCK-8) was chosen to determine cells proliferation. Briefly, SGC-7901 cells were seeded in

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96-well plates at 1×10^4 cells/well, when the cells reached 60 % confluence, the medium was removed and replaced with fresh medium containing varying concentrations of ursolic acid, and incubated for 24, 48, 72 hours. After incubation with culture medium containing CCK-8 reagent for 4 hours, the absorbance was detected at 450 nm using a microplate reader (Biorad, USA). All the experiments were repeated three times.

Flow cytometric analysis for cell apoptosis

SGC-7901 cells were plated in 6-well plates at 4×10^5 per well. Next, cells were collected, washed with cold PBS twice, and gently resuspended in 400 μ l of binding buffer. After the addition of 5 μ l of Annexin V-FITC, the cells were gently vortexed and incubated for 10 min at 4°C in the dark. 10 μ l Propidium iodide (PI) was then added and incubated for another 5 minutes. Flow cytometry was conducted using FACSCalibur flow cytometry (BD Biosciences, USA), following by data analysis with CellQuest software.

Western blot

Whole-cell and cytoplasm proteins from SGC-7901 cells were prepared using a ProteoJET cytoplasmic protein extraction kit (Fermentas, USA). Mitochondrial proteins were isolated using a Mitochondrial Fractionation Kit (Activemotif, China). Proteins were loaded onto SDS-PAGE gels for electrophoresis, transferred onto PVDF membranes, and blocked in 5% BSA prior to incubation with the primary and secondary antibody. The resulting immunocomplexes were visualized by enhanced chemiluminescence. Protein loading was normalized by GAPDH (cytoplasm fraction) and COX IV (mitochondria fraction).

Immunofluorescence

SGC-7901 cells were seeded in a 24-well plate and incubated with ursolic acid. After 24 hours, the cells were incubated with MitoTracker dye (Invitrogen, USA) for 30 minutes. Then, the cells were fixed for 30 minutes with 4% paraformaldehyde in PBS at room temperature, permeabilized, and blocked with 5% nonfat dry milk, 0.5% Triton X-100. Next, the cells were incubated for 2 hours with rabbit cofilin-1 antibody (CST, USA). After washing, FITC-conjugated anti-rabbit IgG antibody (CST, USA) was added for 1 hour. Nucleus was stained with DAPI. Immunofluorescence images were taken with a DMI3000B inverted microscope (Leica, Germany).

Caspase-3 and caspase-9 protease activity analysis

Briefly, SGC-7901 cells were seeded in a 96 well plate and incubated with ursolic acid. After 24 h, 100 μ l of Caspase-Glo 3 or Caspase-Glo 9 reagent (Promega, USA) was added to each well, and the plate was incubated at room temperature for another 2 hours. Luciferase activity of each sample was measured using a TD 20/20 luminometer (Promega, USA). Each sample was measured in triplicate.

Statistical analysis

All the data are expressed as mean $\pm$ standard deviation

($\bar{x} \pm S$). The mean values of two groups were compared by Student's t test, and data analysis was performed with SPSS20 Software. $P < 0.05$ was considered statistically significant.

Results

Ursolic acid promotes the apoptosis of gastric cancer cells SGC-7901

Preliminary study demonstrated that, ursolic acid could significantly inhibit the proliferation of SGC-7901 cells in a dose- and time-dependent manner, with a half inhibition concentration of 35 μ M at 24 hours (Figure 1A). Ursolic acid of 0, 10, 20, and 35 μ M were used to observe its effect on cell apoptosis, and the induced apoptosis of SGC-7901 cells was shown in a dose-dependent manner, with the apoptotic rate 4.68%, 12.44%, 21.73%, and 35.2%, respectively (Figure 1B).

Activation of caspase-3, caspase-9 protease activity and release of cytochrome c into the cytoplasm

Consistent with above findings, the same ursolic acid concentrations with exposure intervals 24 hours caused activation of caspase-3 and caspase-9 (Figure 2A). These events were also accompanied by release of cytochrome c into the cytoplasm from the mitochondria (Figure 2B). The findings implied that ursolic acid might induce the apoptosis of SGC-7901 through the typical mitochondria pathway.

Translocation of cofilin-1 from cytoplasm to mitochondria is involved in ursolic acid promoted apoptosis

Since actin depolymerization factor cofilin-1 played vital effect on cell apoptosis, we next examined the levels of cofilin-1 either in mitochondrial and cytosolic fractions or total cellular extracts using western blot analysis. SGC-

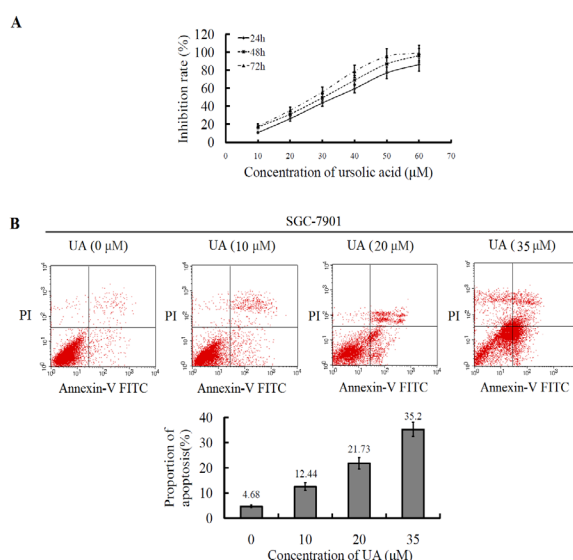


Figure 1. Ursolic Acid Promotes the Apoptosis of Gastric Cancer Cells SGC-7901. A) SGC-7901 cells were treated with ursolic acid of gradual concentrations for 24, 48, 72 hours, and cell survival proportion was analyzed by CCK-8 assay. B) SGC-7901 cells were treated with 0, 10, 20, and 35 μ M ursolic acid for 24 h. Flow cytometry was performed to analyze the apoptotic rate

7901 cells treated with ursolic acid resulted in a marked increase in levels of cofilin-1 in mitochondrial fraction and decrease in levels of cofilin-1 in cytosolic fraction in dose-dependent manners (Figure 3A). However, ursolic acid treatment had little or no effect on the expression of

cofilin-1 in total cellular extract.

All above results suggested that ursolic acid might induce apoptosis of SGC-7901 cells through cofilin-1-mediated mitochondrial apoptosis pathway, wherein the translocation of cofilin-1 from cytoplasm to mitochondria led to the release of cytochrome c from mitochondria to cytoplasm.

To confirm the potential mechanism, cellular localization of cofilin-1 protein was investigated by immunofluorescence. From the representative images, obvious localization of cofilin-1 was observed in the mitochondria of SGC-7901 cells treated with 35 μ M ursolic acid, but in SGC-7901 cells without ursolic acid, little cofilin-1 protein was found in the mitochondria (Figure 3B). Taken together, cofilin-1 might play an important role in the pro-apoptotic process, but its regulatory mechanism needed further investigation.

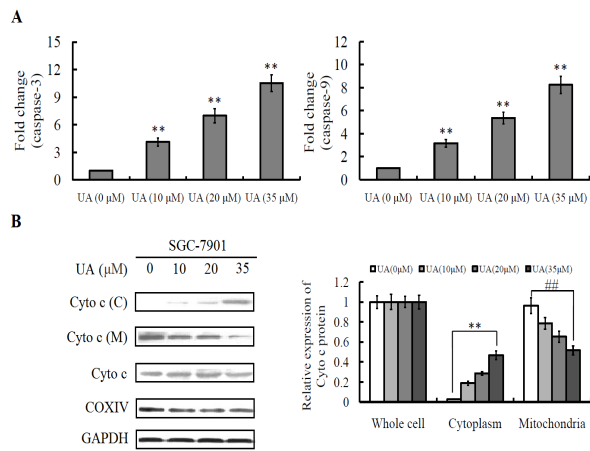


Figure 2. Activation of Caspase-3, Caspase-9 Protease Activity and Release of Cytochrome c into the Cytoplasm. (A) SGC-7901 cells were treated with 0, 10, 20, and 35 μ M ursolic acid for 24 h, caspase-3 and caspase-9 activities were measured, and the data were provided as fold increase in the activities of cells without ursolic acid. (B) GC-7901 cells were treated with 0, 10, 20, and 35 μ M ursolic acid for 24 h, cytoplasmic, mitochondrial, and whole-cell proteins were extracted for western blot analysis. GAPDH and COXIV were used as internal controls for the cytoplasmic and mitochondrial fractions, respectively. ** $p < 0.01$ vs the control cells without ursolic acid treatment

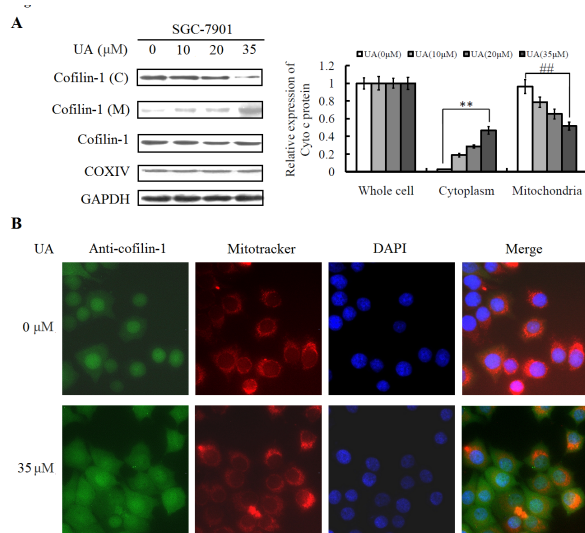


Figure 3. Translocation of Cofilin-1 from Cytoplasm to Mitochondria is Involved in Ursolic Acid Induced Apoptosis. (A) SGC-7901 cells were treated with 0, 10, 20, and 35 μ M ursolic acid for 24 h, the mitochondrial, cytoplasmic, and whole-cell proteins were extracted for detecting the expression and distribution of cofilin-1 protein. ** $p < 0.01$ vs the control cells without ursolic acid treatment. (B) SGC-7901 cells were treated with 0 μ M and 35 μ M ursolic acid for 24 h, then the cells were stained with the mitochondrial marker MitoTracker, followed by immunostaining with anti-cofilin-1 rabbit antibody and FITC-conjugated anti-rabbit secondary antibody. Cell nuclei were stained with DAPI

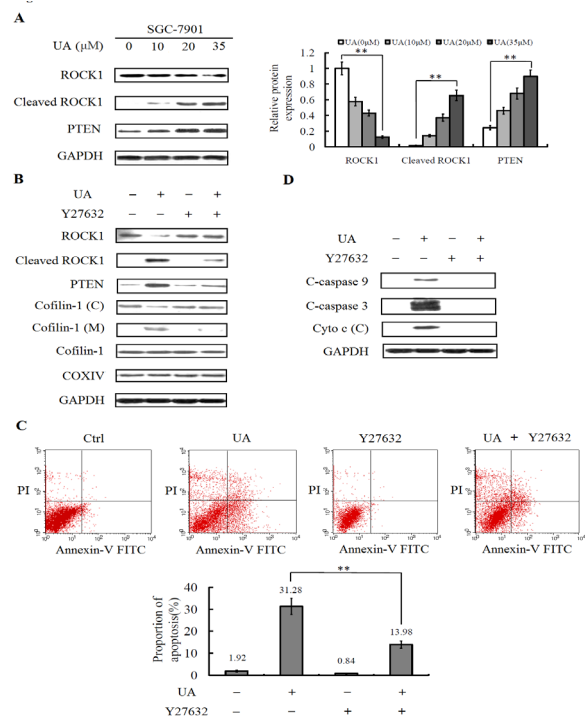


Figure 4. ROCK1/PTEN Signaling Pathway Promotes the Mitochondrial Translocation of Cofilin-1 Protein.

(A) SGC-7901 cells were treated with 0, 10, 20, and 35 μ M ursolic acid for 24 h, the whole-cell proteins were extracted for detecting the expression of ROCK1, cleaved ROCK1 and PTEN proteins. ** $p < 0.01$ vs the control cells without ursolic acid treatment. (B) SGC-7901 cells were pretreated with 20 μ M Y27632, a ROCK1 inhibitor, for 2 h, followed by treating with 35 μ M ursolic acid for 24 h. Mitochondrial, cytoplasmic, and whole-cell proteins were extracted for detecting the expression of ROCK1, cleaved ROCK1, PTEN and cofilin-1 proteins. GAPDH and COXIV were used as internal controls for the cytoplasmic and mitochondrial fractions, respectively. (C) SGC-7901 cells were pretreated with 20 μ M Y27632 for 2 h, followed by treating with 35 μ M ursolic acid for 24 h, and flow cytometry was then performed to analyze the apoptotic rates. ** $p < 0.01$ vs the SGC-7901 cells with only ursolic acid, without 20 μ M Y27632 pretreatment. (D) SGC-7901 cells were pretreated with 20 μ M Y27632 for 2 h, followed by treating with 35 μ M ursolic acid for 24 h, and cytoplasmic proteins were extracted for detecting the expression of cleaved-caspase-9 and cleaved-caspase-3. GAPDH were used as internal controls

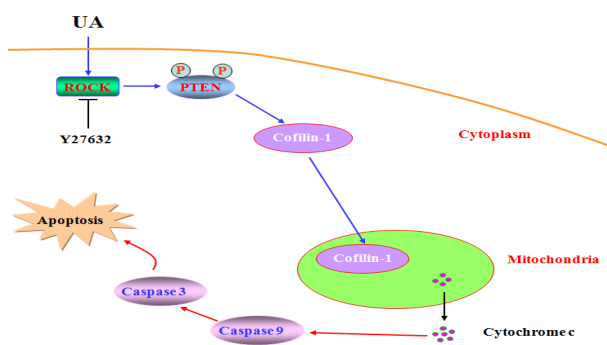


Figure 5. A Hypothetical Illustration for the Pro-Apoptotic Mechanism of Ursolic Acid. UA-induced apoptosis in which ROCK1 activation represents the primary result, leading to PTEN activation, making cofilin translocate to mitochondria, releasing cytochrome c into cytoplasm, activating the activity of caspase-3 and caspase-9, which finally leads to the initiation of apoptosis

ROCK1/PTEN signaling pathway promotes the mitochondrial translocation of cofilin-1 protein

It has been shown that PTEN is a PI3K upstream negative regulator and is regulated by ROCK1 [26]. Next we examined the effect of ursolic acid on the expression of ROCK1 and PTEN. Treating cells with ursolic acid resulted in marked decrease in levels of ROCK1 and increase in cleavage of ROCK1, and marked increase in levels of PTEN in dose-dependent manners (Figure 4A). To determine the functional significance of ROCK1 in regulation of PTEN activity and downstream molecules during UA-induced apoptosis, we then examined the effects of inhibition of ROCK1 by Y27632 on the expression of ROCK1, PTEN. Co-administration of Y27632 significantly blocked UA-mediated ROCK1 activation, PTEN activity. Furthermore, Co-administration of Y27632 significantly blocked UA-mediated cofilin-1 translocation to mitochondria (Figure 4B). We further studied whether inhibition of ROCK1 is sufficient to prevent cells from the effect of ursolic acid on apoptosis. Co-treatment of cells with Y27632 markedly abrogated UA-induced apoptosis (Figure 4C), activation of caspases-3 and -9, and release of cytochrome c (Figure 4D). Taken together, these results demonstrated that ROCK1 played a significant role in regulating the activation of PTEN in response to UA treatment in SGC-7901 cells, which probably contributed to the mitochondrial translocation of cofilin-1 and induction of apoptosis.

Discussion

Gastric cancer, one of the worldwide malignant tumours, its occurrence is tightly associated with excessive proliferation (Sugimura et al., 1975; Chen et al., 2013), abnormal differentiation (Aizawa et al., 1999), reduced apoptosis (Ikeda et al., 1998), and so on. Ursolic acid, as one of the extracts from traditional Chinese medicine bearberry, exerts much effects including growth inhibition and apoptosis-inducing on various tumour cells such as gastric cancer BGC-803 cells (Wang et al., 1975), colorectal cancer HT29 cells (Anderson et al., 2003), human multiple myeloma U266 cells (Pathak et al., 2007).

Cofilin is a member of ADF/cofilin family (Chen et al., 2010), it plays many important parts in apoptosis (Chua et al., 2003; Wang et al., 2008), differentiation (Zhou et al., 2012), invasion and migration (Jang et al., 2012; Popow-Wozniak et al., 2012), tumorigenesis (Polachini et al., 2012). Cofilin was found to translocate to mitochondria after staurosporine-induced apoptosis in neuroblastoma cells (Chua et al., 2003), or oxidant-induced apoptosis in neutrophils (Klamt et al., 2009). These results demonstrate that cofilin is crucial for its apoptosis inducing activity. Mitochondrial translocation of cofilin may affect mitochondria function, result in the release of cytochrome c, and induce cell apoptosis (Chua et al., 2003; Rehklau et al., 2012).

Recent evidence revealed that PTEN (Xu et al., 2012) is a newly identified ROCK substrate, which is involved in the regulation of cell death and survival (Li et al., 2005). Consistence with this report, our findings suggest that activation of ROCK1 and PTEN contribute to UA-induced mitochondrial translocation of cofilin from cytoplasm to mitochondria, release of cytochrome c, activation of caspase-3, caspase-9, and finally lead to the apoptosis of gastric cancer SGC-7901 cells. Specifically, UA resulted in activation of ROCK1 and PTEN, but when the ROCK1 activity was inhibited by Y27632, a ROCK1 inhibitor, UA-mediated apoptosis was attenuated through preventing PTEN activity, and mitochondrial translocation of cofilin-1.

In conclusion, our findings suggest a detailed mechanism in UA-induced apoptosis in which ROCK1 activation represents the primary result, leading to PTEN activation, making cofilin-1 translocate to mitochondria, releasing cytochrome c into cytoplasm, activating the activity of caspase-3 and caspase-9, which finally leads to the initiation of apoptosis (Figure 5). Therefore, ROCK1/PTEN mediated mitochondrial translocation of cofilin-1 played a crucial role in UA-inducing apoptosis, and it might provide an important strategy for treatment of patients with gastric cancer.

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