雷公藤红素通过抑制PI3K/Akt信号通路诱导食管癌细胞凋亡

侯尧[1] 裴蓉[2] 徐佳[1] 陈静[1] 伍春莲[1]*

([1]西华师范大学, 西南野生动植物资源保护教育部重点实验室, 南充 637009;
[2]成都中医药大学公共卫生学院, 成都 611137)

摘要 越来越多的研究表明, 雷公藤红素可以诱导细胞凋亡, 但是其对食管癌细胞的作用尚未可知。该研究通过体外实验探讨了雷公藤红素对食管癌ECA-109细胞增殖和凋亡的影响, 结果显示雷公藤红素对细胞增殖的抑制有明显的剂量依赖性, 且在高浓度(≥1.0 μmol/L)时细胞增殖受到明显抑制。雷公藤红素处理显著增加了Bax和p53的mRNA和蛋白表达水平, 同时抑制了Bcl-2的表达以及Akt、NF-κB-p65、PDK1和PTEN的磷酸化。这些结果揭示了雷公藤红素影响食管癌细胞增殖和凋亡的潜在机制, 同时也说明了雷公藤红素在治疗食管癌方面有潜在的应用价值。

关键词 雷公藤红素; 食管癌; PI3K/Akt信号通路

Celastrol Induces Apoptosis of Esophagus Cancer Cells by Inhibiting PI3K/Akt Signaling Pathway

HOU Yao[1], PEI Rong[2], XU Jia[1], CHEN Jing[1], WU Chunlian[1]*

([1]*Key Laboratory of Southwest China Wildlife Resources Conservation (China West Normal University), Ministry of Education, Nanchong 637009, China*; [2]*School of Public Health, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China*)

Abstract Increasing evidence indicates that Cel (celastrol) promotes apoptosis. However, the effects of celastrol on EC (esophageal cancer) have not been investigated. This study investigated the effect of celastrol on esophageal cancer ECA-109 cell proliferation and apoptosis *in vitro* and it revealed that cell proliferation was inhibited by celastrol in dose-dependent manner and was significantly inhibited at high concentrations (⩾ 1.0 μmol/L). Treatment with celastrol significantly increased the mRNA and protein expression of Bax and p53, while the expression of Bcl-2 and the phosphorylation of Akt, NF-κB-p65, PDK1 and PTEN were inhibited. These findings reveal a potential mechanisms by which celastrol affects the proliferation and apoptosis of esophageal cancer cells and suggest that celastrol has potential application value in the treatment of esophageal cancer.

Keywords celastrol; esophageal cancer; PI3K/Akt signaling pathway

EC (esophageal cancer) is the eighth most common cancer worldwide, with about 407 000 deaths (5.4% of all cancer deaths) every year[1]. Esophageal cancer is the seventh most common cancer and the sixth leading cause of death worldwide, with esophageal squamous cell carcinoma accounting for 90% of

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*通讯作者。Tel: 18990874796, E-mail: wcl_xj@163.com
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*Corresponding author. Tel: +86-18990874796, E-mail: wcl_xj@163.com

esophageal cancer cases[2-3]. The incidence of esophageal cancer is on the rise globally, with about 80% of the cases occurring in developing countries, and the incidence rate in males is 3 times of that in females[4]. According to statistics from the National Cancer Center, China currently has the highest incidence and mortality rate of esophageal cancer in the world, with more than 80% of new cases of esophageal cancer occurring in China. Shanxi, Henan, Hebei and other mountainous areas of Taihang are the new high incidence areas of esophageal cancer[5-6]. In 2018, there were 307 359 new cases of esophageal cancer and 283 433 deaths in China[7]. Therefore, esophageal cancer has become one of the main malignant tumors threatening the health of Chinese residents. Although the incidence of esophageal cancer in China has declined in the past 20 years, esophageal cancer is still a huge burden[8]. Carcinoma of the esophagus is clinically characterized by rapid advance, a tendency for metastasis and recurrence, and poor prognosis. Therefore, new techniques for treating carcinoma of the esophagus, including the development of effective drugs, are increasingly required.

There are two main signaling pathways that regulate apoptosis, namely receptor mediated apoptosis pathway (intrinsic pathway) and mitochondria mediated apoptosis pathway (mitochondrial pathway). The mitochondria mediated apoptosis pathway, which is mainly dependent on mitochondria and regulated by mitochondrial proteins (mainly Bcl-2 family proteins), mainly responds to stress signals generated inside cells. There are three main groups of Bcl-2 family proteins: the pro-survival family members, the pro-apoptotic BAX/BAK family members and pro-apoptotic BH3-only proteins. Bcl-2 belongs to the anti-apoptotic protein family, and Bax belongs to the pro-apoptotic protein family[9-10]. Many papers report that Bcl-2 pathway, as the main signal transduction pathway mediated by cytokine receptors, is widely involved in tumor cell proliferation, differentiation and overcoming, which has an important impact on the body[11-12]. Bcl-2 is a specific inhibitor of apoptosis in cancer cells, and the increase of Bcl-2 expression can significantly promote the proliferation activity of cancer cells[13]. Bax protein can make the mitochondrial outer membrane permeable, resulting in mitochondria-mediated apoptosis. Bax is a key of mitochondrial apoptosis[14]. Therefore, Bcl-2 and Bax have become the therapeutic targets of cancer.

p53 is a tumor suppressor gene whose mutation can be detected in more than half of cancers[15]. As a transcription factor, *p53* induces the expression of various genes associated with apoptosis, cell cycle arrest, and DNA repair[16]. In addition, p53 is involved in biological processes such as maintaining genomic stability[17-18], maintaining epigenetic stability[19], and suppressing carcinogenic signals[20].

Natural drug have been an important source of anti-tumor drug due to potent pharmacological effects and better toxicology profiles. In recent years, researchers have paid close attention to identifying bioactive constituent from medicinal plants. Celastrol is a robust bioactive compound derived from *Tripterygium wilfordii*, *Celastrus orbiculatus*, and others that belong to the *Celastraceae* family[21]. Celastrol is a pentacyclic triterpene that belongs to a small class of organic compounds called quinone methides. It has a molecular weight of 450.6 and its molecular formula is $C_{29}H_{38}O_4$[22]. Recent studies have shown that *Tripterygium wilfordii* has anti-inflammatory, anti-immune, and anti-tumor effects[23-24]. At the same time, celastrol can induce apoptosis in human triple negative breast cancer cells by up-regulating Bax expression and down-regulating PI3K enzyme activity and Akt phosphorylation[25].

In this study, we used MTT analysis and DAPI staining in ECA-109 cells to study the effects of celastrol on proliferation, morphology, and proliferative activity of esophageal cancer cells. We also explored the possible molecular mechanisms of celastrol inducing apoptosis. Our results indicate that celeastrol inhibits ECA-109 cell proliferation and induces apoptosis by inhibiting Bcl-2 expression and Akt and NF-κB signaling pathways. These findings provide a better explanation for the mechanism of celastrol-induced apoptosis and provide a theoretical and experimental basis for the clinical use of celastrol to treat esophageal cancer.

1 Materials and methods

1.1 Reagents and antibodies

Celastrol with 98% or higher purity was purchased from Shanghai Yuanye Biological Technology (Shanghai, China). Celastrol was dissolved at a concentration of 50 mmol/L in dimethyl sulfoxide (DMSO) as a stock solution (stored at –20 °C). It was then further diluted in cell culture medium to create working concentrations. The maximum final concentration of DMSO was less than 0.1% for each treatment, and was also used as a control.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma (St. Louis, MO). Rabbit antibodies against human Bax, Bcl-2, p53, Akt, Phospho-Akt, NF-κB-p65, Phospho-NF-κB-p65, Phospho-PDK1 and Phospho-PTEN were obtained from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody GAPDH (as internal loading control) was purchased from Invitrogen (Carlsbad, CA). RPMI-1640 was purchased from Gibco and a SYBR® Premix Ex Taq™ Kit was purchased from TaKaRa.

1.2 Cell culture

The esophagus cancer cells (ECA-109 cells) was used in this study. ECA-109 cells were cultured, as is routine, in RPMI-1640 medium supplemented with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin at 37 °C in an incubator with 5% CO_2. The cells were provided with fresh medium every 2 to 3 days. Cells were then incubated with different concentrations of celastrol for 24 and 48 h.

1.3 Cell viability assay

The viability of the cell proliferation was determined by MTT assay. The trypsinized cells were plated in 96-well plates at a density of 4×10^3 cells/each well for 24 h. Cells were exposed to different concentrations of celastrol for 24 or 48 h in an incubator with 5% CO_2 at 37 °C. At the end of treatment, 10 μL of 0.5% MTT was added to the medium and incubated for 4 h at 37 °C. The supernatant was removed and 0.2 mL DMSO was used to dissolve the precipitate. Absorbance was measured at a wavelength of 492 nm in a plate reader. The independent experiments were each run three times.

1.4 Flow cytometry

The cells were pretreated with a series of concentrations of celastrol for 24 h. The cells were then harvested, washed with PBS, dealed with Annexin V-FITC kit, and detectd by flow cytometry.

1.5 DAPI Staining

The cells were pretreated with a series of concentrations of celastrol for 24 h. The cells were then harvested, washed with PBS, and added with DAPI (4’,6-2 amidine base-2-phenyl indole) in darkness. Incubate at room temperature for 5-10 minutes, shielded from light, cleaning 4-5 times with PBS. Then, the ECA-109 cells were observed under a fluorescence microscope.

1.6 AO/PI staining

The cells were pretreated with a series of concentrations of celastrol for 24 h. The cells were then harvested, washed with PBS. AO/PI was added in darkness. At room temperature, cells were incubated 5-10 s away from light. Then, the ECA-109 cells were photographed under a fluorescence microscope.

1.7 Assessment of changes in the mitochondrial membrane potential

The cells were pretreated with a series of concentrations of celastrol for 24 h. The cells were then harvested, washed with PBS, treated with JC-1 kit, and detected by flow cytometry and took a photograph under a fluorescence microscope.

1.8 qRT-PCR (quantitative real-time polymerase chain reaction) analysis

For RNA isolation, total RNA from ECA-109 cells for use in qRT-PCR was extracted using Trizol reagent in accordance with the manufacturer’s protocol (TaKaRa). The RNA purity and concentration were determined by eppendorf Biophotometer and RNA quality was identified using electrophoresis. One micrograms total RNA was used as template to synthesis cDNA with TaKaRa PrimeScript™ RT reagent kit. PCR was detected by SuperReal PreMix Plus (SYBR Green) according to the manufacturer’s recommended protocol.

qRT-PCR was performed using a SYBR® Premix

表1 基因表达转录本qRT-PCR引物

Table 1 Primers for qRT-PCR analysis of gene transcript expression

基因 Gene	上游引物 Forward primer	下游引物 Reverse primer
Bax	5′-CGA TTC ATC TAC CCT GCT GAC CT-3′	5′-CTT GAG CAA TTC CAG AGG CAG T-3′
Bcl-2	5′- GGA TAA CGG AGG CTG GGA TG-3′	5′- TTG TGG CTC AGA TAG GCA CC-3′
p53	5′-AGC ACT GTC CAA CAA CAC CA-3′	5′-CTT CAG GTG GCT GGA GTG AG-3′
GAPDH	5′-CAT CAA GAA GGT GGT GAA GCA G-3′	5′-AAA GGT GGA GGA GTG GGT GTC-3′

Ex Taq™ Kit (TaKaRa) with forward and reverse primers for *Bax*, *Bcl-2*, *p53* and *GAPDH* (Table 1).

1.9 Western blot analysis

Total proteins were extracted by lysis buffer. The protein concentration in the supernatants was detected using a BCA protein assay. Equal amounts of protein were prepared and separated by SDS-PAGE and transferred onto the NC (nitrocellulose) membranes. The membrane was blocked with 5% non-fat milk in TBST at 37 °C for 1.5 h and incubated with indicated antibodies overnight at 4 °C. After extensive washing with TBST buffer, the blots were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. After extensive washing with TBST buffer, target proteins were detected by enhanced chemiluminescence reagents ECL.

1.10 Statistical analysis

All experimental data were presented as $\bar{x}\pm s$, and results were analysed by using SPSS (statistical package for social sciences) (IBM Incorporation) version 24.0 software. $P<0.05$ were considered statistically significant.

2 Results

2.1 Celastrol inhibits cell proliferation of human ECA-109 cells

In this study, we determined the effect of celastrol on ECA-109 cells proliferation by treating cells with celastrol at different concentration gradients (0, 0.5, 1.0, 2.0, 4.0, 6.0 μmol/L) for 24 and 48 h. In comparison with that of a solvent control (DMSO), after treatment with celastrol for 24 h, the viability of ECA-109 cells was significantly affected at a concentration of more than 1 μmol/L ($P<0.01$) (Fig.1B). When ECA-109 cells were treated with celastrol for 48 h, the data indicated that ECA-109 cell proliferation was significantly inhibited at concentrations greater than 0.5 μmol/L ($P<0.05$). It indicated that celastrol inhibited cell viability in a dose- and time-dependent manner. This concentration range was then used in all subsequent experiments.

2.2 Celastrol-induced apoptosis

We examined the effect of celastrol inducing apoptosis of ECA-109 cells. As shown in Fig.1, after ECA-109 cells were treated with celastrol (0.5 and 1.0 μmol/L) for 24 h, apoptotic cells and death cells were significantly increased in a dose-dependent manner ($P<0.05$, $P<0.01$) compared with the control; apoptotic cells were increased to 31.2% and 36.2%, respectively (Fig.1C).

2.3 Celastrol-induced morphologic changes of apoptosis

It is well known that alternation of cell morphology, apoptotic body formation, and DNA condensation are the hallmarks of apoptosis. To confirm celastrol-induced apoptosis, ECA-109 cells were subjected to DAPI staining. Control cells presented intact, complete nucleus karyotype and evenly distributed chromatin (Fig.1D: A1). With the increase of concentration of celastrol, the chromatin condensed and gathered to the peripheral when the concentration was 0.5 μmol/L (Fig.1D: A2). When the concentration reached 1 μmol/L, the chromatin condensed further and formed a lot of particulate matter, ultimately, presented the nucleus rupture and collapse (Fig.1D: A3).

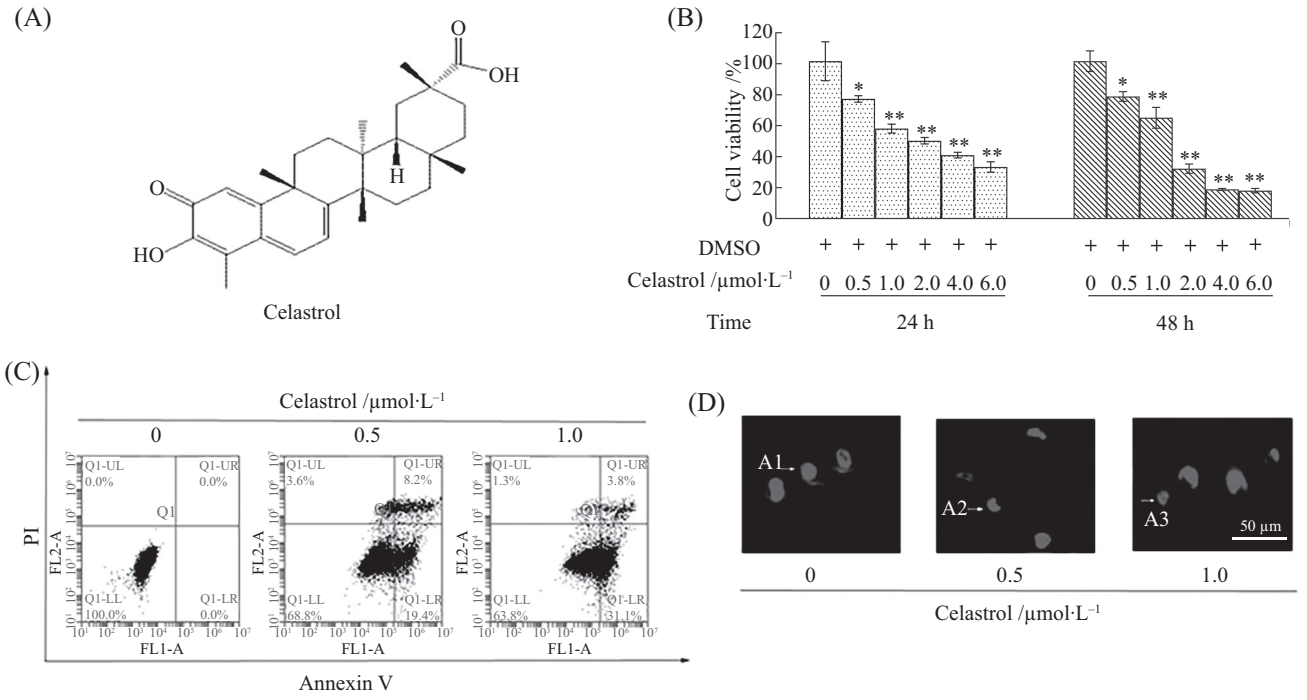
2.4 AO (acridine orange)/PI (propidium iodide) staining

Fluorescent microscopy was carried out to observe any morphological changes and apoptotic fea-

tures of normal untreated and treated ECA-109 cells by means of AO and PI staining. AO is membrane-permeable and stains the cell nuclei green, indicating the cells are viable. However, the intercalating dye PI is membrane-impermeable, thus only taken up by non-intact cells and stains the nuclei red. As shown in Fig.2A, the nuclei of untreated normal cells are stained green and show normal structure, while celastrol-treated cells are stained red and orange, representing the hallmark of apoptosis. With the increase of concentration of celastrol, orange fluorescence was increased in a dose-dependent manner.

2.5 Mitochondrial membrane potential (Dym) estimation

Fluorescent microscopy and flow cytometer were carried out to observe any morphological changes and apoptotic features of normal untreated and treated ECA-109 cells by means of JC-1. When mitochondrial membrane potential is high, JC-1 gathered in the mitochondrial matrix forming polymer, can produce red fluorescence; When the mitochondrial membrane potential was low, JC-1 did not aggregate in the mitochondrial matrix to form monomer and produced green fluorescence. As shown in Fig.2, with the increase of concentration of celastrol, red fluorescence is reduced and the green fluorescence is increased. When the concentration of celastrol is 0 μmol/L, the green fluorescence is only 18.7%. When the concentration of celastrol is 1.0 μmol/L, the green fluorescence reaches 75.4% (Fig.2B). Fluorescent staining is found that green fluorescence is increased, and there are dose-



A: 雷公藤红素的化学结构。B: 不同浓度(0、0.5、1.0、2.0、4.0、6.0 μmol/L)的雷公藤红素处理ECA-109细胞24 h和48 h对ECA-109细胞增殖的影响。C: 不同浓度(0、0.5、1.0 μmol/L)的雷公藤红素处理ECA-109细胞24 h。Annexin V-FITC试剂盒检测细胞凋亡。D: 不同浓度(0、0.5、1.0 μmol/L)的雷公藤红素处理ECA-109细胞24 h。DAPI染色观察雷公藤红素对ECA-109细胞凋亡形态学上的影响。图中A1、A2和A3箭头指向细胞核。*P<0.05, **P<0.01, 与对照组(DMSO)相比。

A: the chemical structure of celastrol. B: effect of celastrol on ECA-109 cells proliferation treated with various concentrations (0, 0.5, 1.0, 2.0, 4.0 and 6.0 μmol/L) of celastrol for 24 and 48 h. C: ECA-109 cells were treated with celastrol at various concentrations (0, 0.5, 1.0 μmol/L) for 24 h. Cell apoptosis was determined by Annexin V-FITC kit. D: ECA-109 cells were treated with celastrol at various concentrations (0, 0.5, 1.0 μmol/L) for 24 h. DAPI staining was used to observe the morphological effects of tripterine on apoptosis of ECA-109 cells. The A1, A2, and A3 arrows point to the nucleus. *P<0.05, **P<0.01 compared with control group (DMSO).

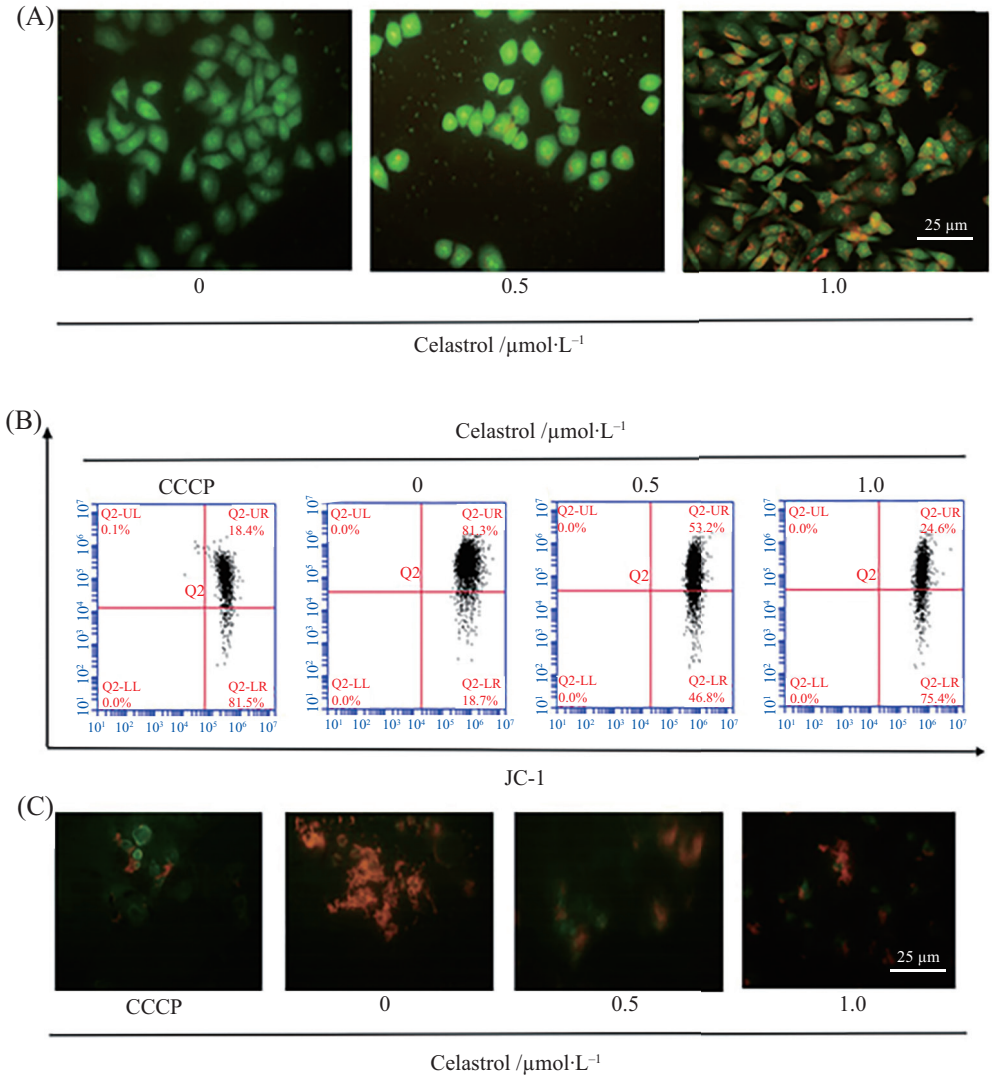
图1 雷公藤红素抑制ECA-109细胞的增殖

Fig.1 Celastrol inhibits ECA-109 cell proliferation

dependent (Fig.2C).

2.6 Celastrol inhibits Bax, Bcl-2 and p53 mRNA and protein expression in human ECA-109 cells

To determine whether celastrol suppresses Bax/Bcl-2 and p53 expression, qRT-PCR and Western blot were used to examine the expression of Bax/Bcl-2 and p53 at the mRNA and protein levels. Total RNA and protein were isolated from ECA-109 cells treated with vehicle (control group), 0.5 μmol/L (Cel-0.5 group) and 1.0 μmol/L (Cel-1.0 group) celastrol for 24 h. As shown in Fig.3, celastrol significantly increased the expression of Bax and p53 and inhibited the expression of Bcl-2. Celastrol can affect the expression of Bax/Bcl-2 and p53. Compared with the control group, experimental groups starting from 0.5 μmol/L(P<0.05, P<0.01) have a significant effect.

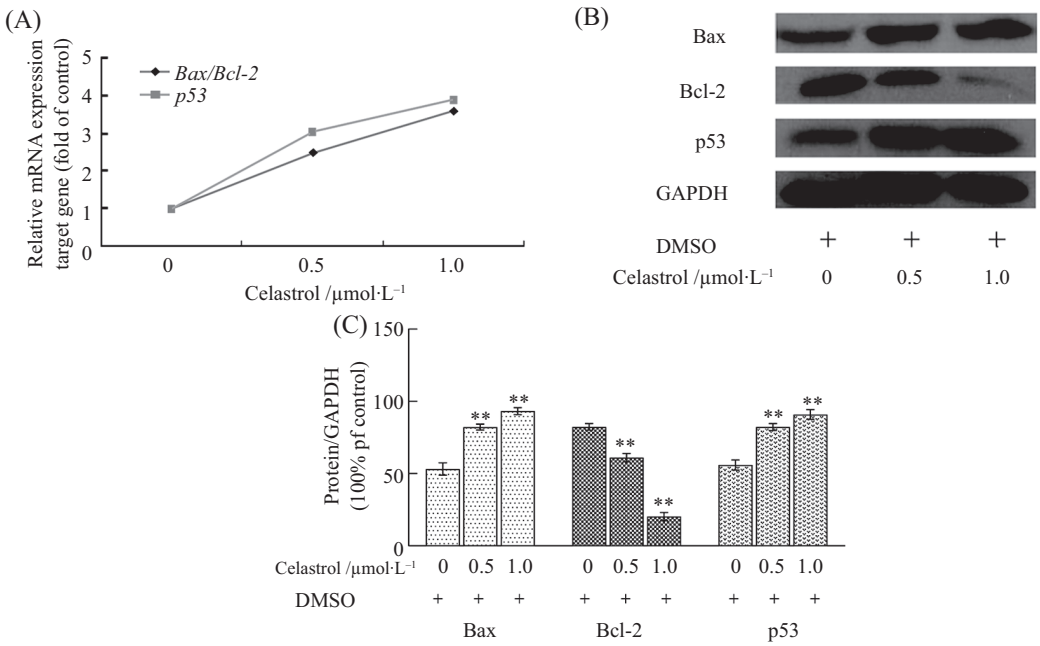


A: 不同浓度(0、0.5、1.0 μmol/L)雷公藤红素处理ECA-109细胞24 h, AO/PI染色检测雷公藤红素对ECA-109细胞凋亡形态学变化的影响。B: 不同浓度(0、0.5、1.0 μmol/L)雷公藤红素处理ECA-109细胞24 h, JC-1试剂盒检测细胞凋亡。CCCP可以作用于线粒体, 改变H^+通透性, 通过线粒体途径诱导凋亡。这里CCCP作为对照组。C: JC-1染色后检测不同浓度(0、0.5、1.0 μmol/L)雷公藤红素处理ECA-109细胞24 h对ECA-109细胞凋亡形态学上的影响。

A: effect of celastrol was detected on morphologic changes of apoptosis in ECA-109 cells by AO/PI staining after treated with celastrol at various concentrations (0, 0.5, 1.0 μmol/L) for 24 h. B: ECA-109 cells were treated with celastrol at various concentrations (0, 0.5, 1.0 μmol/L) for 24 h, cell apoptosis was determined by JC-1 kit. CCCP can act on mitochondria, change H^+ permeability, and induce apoptosis through mitochondrial pathway. Here CCCP serves as the control group. C: effect of celastrol on morphologic changes of apoptosis in ECA-109 cells was detected by JC-1 staining after treated with celastrol at various concentrations (0, 0.5, 1.0 μmol/L) for 24 h.

图2 雷公藤红素诱导ECA-109细胞凋亡

Fig.2 Celastrol induces apoptosis in ECA-109 cell



A: 不同浓度(0、0.5、1.0 μmol/L)雷公藤红素处理ECA-109细胞24 h后, qRT-PCR检测*Bax/Bcl-2*和*p53*的mRNA表达。B: 不同浓度(0、0.5、1.0 μmol/L)雷公藤红素处理ECA-109细胞24 h后, Western blot检测Bax、Bcl-2和p53的表达。C: 统计分析Bax、Bcl-2和p53的表达情况。GADPH用作内参对照。*P<0.05, **P<0.01, 与对照组(DMSO)相比。

A: qRT-PCR assay was used to examine the mRNA expression of *Bax/Bcl-2* and *p53* after treated with celastrol at various concentrations (0, 0.5, 1.0 μmol/L) for 24 h. B: Western blot assay was used to examine the protein expression of Bax, Bcl-2 and p53 after treated with celastrol at various concentrations (0, 0.5, 1.0 μmol/L) for 24 h. C: statistical analysis of the protein expression of Bax, Bcl-2 and p53. GAPDH was used as an internal loading control. *P<0.05, **P<0.01 compared with control group (DMSO).

图3 雷公藤红素对ECA-109细胞中Bcl-2、Bax和p53表达的影响

Fig.3 Effect of cleastrol on Bcl-2, Bax and p53 expression in ECA-109 cells

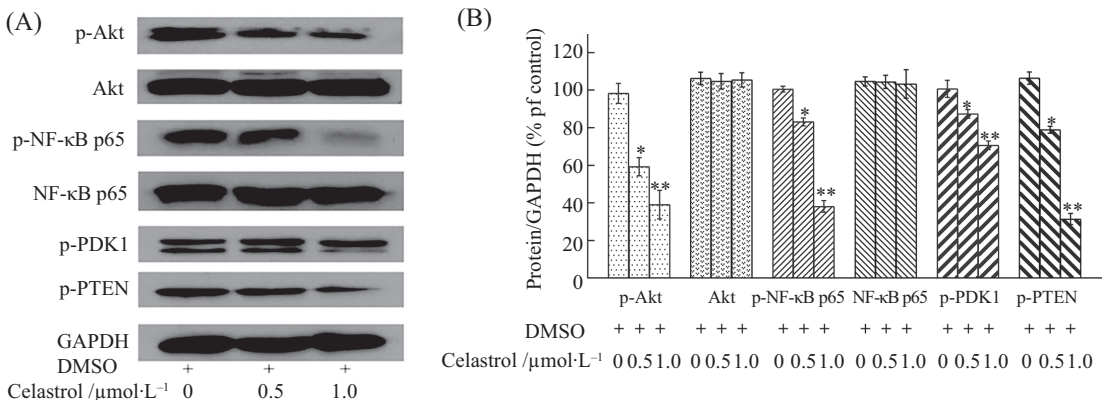
2.7 Effects of celastrol on the Akt and NF-κB pathway

To further explore the effect of celastrol on signaling pathways, Western blot was used to examine the expression of Akt, NF-κB-p65, PDK1 and PTEN in ECA-109 cells treated with various concentrations (0, 0.5, 1.0 μmol/L) of celastrol for 3 h. *PDK1* is an upstream gene of Akt and plays an important role in regulating PI3K/Akt signaling pathway. PDK1-mediated PI3K/Akt signaling pathway is closely related to the occurrence and development of tumors[25]. PTEN is an upstream inhibitor of PI3K/Akt signal transduction pathway which negatively regulates the PI3K/AKT signaling pathway, inhibits cell growth and accelerates cell apoptosis[26]. As shown in Fig.4A and Fig.4B, celastrol can significantly inhibit the phosphorylation of Akt, NF-κB-p65, PDK1 and PTEN in a concentration-dependent manner, but have no effect on the expression of Akt and NF-κB-p65. These results suggest that celastrol can inhibit the PI3K/Akt signaling pathway and influence NF-κB signaling pathway by decreasing phosphorylation of Akt, NF-κB-p65, PDK1 and PTEN.

3 Discussion

In this study, we demonstrated that celsatrol could inhibit ECA-109 cells proliferation and induce apoptosis. Moreover, qRT-PCR and Western blot analysis confirmed that celastrol down-regulated Bax/Bcl-2 and p53 expression. We further revealed that celastrol repressed the phosphorylation of Akt, NF-κB-p65, PDK1 and PTEN to decrease activations of Akt, NF-κB-p65, PDK1 and PTEN. Therefore, these findings highlight the significance of celastrol in suppressing human esophageal carcinoma cell proliferation and promoting cell apoptosis through inhibiting Bax/Bcl-2 and p53 expression and the Akt and NF-κB signaling pathway.

Cell proliferation, differentiation and apoptosis



A: 不同浓度(0、0.5、1.0 μmol/L)雷公藤红素处理ECA-109细胞24 h后、Western blot检测Akt、磷酸化的Akt、磷酸化的NF-κB-p65、NF-κB-p65、磷酸化的PDK1和磷酸化的PTEN的表达情况。B: 统计分析蛋白表达。*P<0.05, **P<0.01, 与对照组(DMSO)相比。

A: Western blot assay was used to examine the protein expression of Akt、pAkt、p-NF-κB-p65、NF-κB-p65、p-PDK1 and p-PTEN after treated with celastrol at various concentrations (0、0.5、1.0 μmol/L) for 24 h. B: statistical analysis of protein expressions. *P<0.05, **P<0.01 compared with control group (DMSO).

图4 雷公藤红素对ECA-109细胞内Akt和NF-κB信号通路的影响

Fig.4 Effects of celastrol on the Akt and NF-κB pathway in ECA-109 cells

imbalance are the important factors of the tumor. Induction of tumor cell apoptosis is a new way of tumor treatment research. Down-regulation of Bcl-2 expression is a key factor during apoptosis. Bcl-2 affects tumor cell apoptosis, through inhibiting signaling pathways including Ros[27], NF-κB[28] and Akt[29] pathways. Due to Bcl-2 inhibiting tumor cell apoptosis, Bcl-2 might be used as a novel therapeutic target of cancer. *p53* is an apoptosis activation gene and plays an important role in the process of programmed cell death, and it can induce cell cycle arrest, senescence, and differentiation[16-20]. Inactivation of p53 function is a pivotal aspect of tumor formation in a broad spectrum of human cancers[15]. As a result, maintaining and enhancing p53 function will help to improve the current cancer treatment effect.

So far, a variety of studies have shown that celastrol may be used as an effective anti-inflammatory and therapeutic anti-tumor drug[30-31]. Previous studies have shown celastrol to be effective against a variety of cancers, such as breast cancer, melanoma and squamous cell carcinoma, and prostate cancer. In this study, we found that proliferation of ECA-109 cell was significantly inhibited by 1.0 μmol/L celastrol (Fig.1).

Cancer cell shows limitless proliferation and activates intracellular signaling pathways. Our results showed that more than 1.0 μmol/L of celastrol significantly promoted cell apoptosis in a dose-dependent manner (Fig.1). Mitochondrial membrane potential drop is a symbol of early apoptosis. The decrease of membrane potential can be easily detected by the fluorescence shift from red to green of JC-1, and the fluorescence shift from red to green of JC-1 can also be used as an indicator of early apoptosis. Our results suggest that less than 1.0 μmol/L celastrol significantly reduced mitochondrial membrane potential in a dose-dependent manner (Fig.2). Bax and Bcl-2 play important roles in esophagus cancer ECA-109 cell. Down-regulation of Bcl-2 expression and up-regulation of Bax expression can cause ECA-109 cell apoptosis. We confirmed that celastrol could inhibit expression of Bcl-2 and induce expressions of p53 and Bax in ECA-109 cells (Fig.3). Therefore, inhibition of Bcl-2 expression and up-regulation of p53 and Bax expression can induce cancer cell apoptosis. In addition, we also found that celastrol reduced Akt, NF-κB-p65, PDK1 and PTEN phosphorylation when they induced cell apoptosis (Fig.4). The PI3K/Akt signaling cascade is frequently disrupted in many human cancers, and it is a key player in mediating tumor cell survival and escape from apoptosis.

In summary, we demonstrated that celastrol was able to suppress esophagus cancer ECA-109 cell proliferation and induce its apoptosis *in vitro* by regulat-

ing Bax/Bcl-2 expression and inhibiting PI3K/Akt signaling pathway. Our study provides new evidence that celastrol may be able to serve as an efficient anti-metastatic drug in the treatment of esophagus cancer.

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