RESEARCH ARTICLE

Emodin-Provoked Oxidative Stress Induces Apoptosis in Human Colon Cancer HCT116 Cells through a p53-Mitochondrial Apoptotic Pathway

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Abstract

    Emodin, a natural anthraquinone isolated from the traditional Chinese medicine *Radix rhizoma* Rhei, can induce apoptosis in many kinds of cancer cells. This study demonstrated that emodin induces apoptosis in human colon cancer HCT116 cells by provoking oxidative stress, which subsequently triggers a p53-mitochondrial apoptotic pathway. Emodin induced mitochondrial transmembrane potential loss, increase in Bax and decrease in Bcl-2 expression and mitochondrial translocation and release of cytochrome c to cytosol in HCT116 cells. In response to emodin-treatment, ROS increased rapidly, and subsequently p53 was overexpressed. Pretreatment with the antioxidant NAC diminished apoptosis and p53 overexpression induced by emodin. Transfecting p53 siRNA also attenuated apoptosis induced by emodin, Bax expression and mitochondrial translocation being reduced compared to treatment with emodin alone. Taken together, these results indicate that ROS is a trigger of emodin-induced apoptosis in HCT116 cells, and p53 expression increases under oxidative stress, leading to Bax-mediated mitochondrial apoptosis.

Keywords: Emodin - Human colon cancer HCT116 cells - ROS - p53 - Mitochondrial apoptosis

Introduction

Colorectal cancer is one of the most common cancers globally. In 2012, the worldwide incidence number was estimated to be 1,361,000, the third most next to lung and breast cancer. Almost 55% of the cases occur in more developed regions, but the mortality was higher in less developed regions (52.02%) that in more developed regions (47.98%) (International Agency for Research on Cancer, 2014a). In China, along with the changes in diet and life style, the ASR (age-standardised rate) of incidence and mortality has increased to 14.2 and 7.4 per 100,000 respectively(International Agency for Research on Cancer, 2014b).

Emodin (1, 3, 8-trihydroxy-6-methyl-anthraquinone) is an anthraquinone existing in many kinds of traditional Chinese medicine such as RHEI RADIX ETRHIZOMA, POLYGONI CUSPIDATI RHIZOMA ET RADIX and POLYGONIMUUI MULTIFLORI RADIX(China Pharmacopoeia Committee, 2010). Emodin has anti-cancer effect based on inhibiting cell proliferation, inducing apoptosis, suppressing migration, invasion and angiogenesis (He et al., 2012; Lin et al., 2012; Manu et al., 2013; Subramaniam et al., 2013). It has been demonstrated that emodin suppresses tumor growth in tumor nude mice xenografts bearing human colon cancer LS1034 cells and induce LS1034 cells apoptosis in vitro (Ma et al., 2012). And emodin could inhibit VEGF-receptor phosphorylation in human colon cancer HCT116 cells (Lu et al., 2008). However, the molecular mechanisms of emodin-induced apoptosis in colorectal cancer cells need more investigation.

Many reports have indicated that apoptosis induction of emodin is at least partly dependent on ROS upregulation (Su et al., 2005; Lai et al., 2009; Lin et al., 2009; Huang et al., 2013). Our study is a preliminary investigation on the signal pathway and the role of ROS in emodin-induced apoptosis in HCT116 cells.

Materials and Methods

Cell culture and treatment

HCT116 cells were cultured in RPMI 1640 medium (Gibco/BRL, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Gibco/BRL), 100units/ml penicillin, and 100mg/l streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Emodin (Aladdin, Shanghai, China) dissolved in DMSO as a 40mmol/l stock

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solution was used for the treatment of cells. In solvent control group, cells were treated with 0.1% DMSO. For the NAC (N-acetylcysteine, Beyotime, Shanghai, China) pretreatment, 5mmol/l NAC was treated one hour before emodin.

Cell viability assay

Cells were treated with emodin at concentrations from 0 to 320μmol/l for 48 h. Cell viability was analyzed by MTT (SunshineBio, Nanjing, Jiangsu, China) assay. 20μl of MTT(5mg/ml) was added to each well, and then microplates were kept at 37°C in 5% CO_2 for 4 h. The optical density (OD) of each sample was measured at 570nm on automatic plate reader (Bio Rad, Hercules, California, USA).

Apoptosis assay

Cell apoptosis was detected by the Annexin V-FITC/ PI Apoptosis Detection Kit (KeyGEN BioTECH, Nanjing, Jiangsu, China) according to the manufacturer's instruction. Cells were washed twice with PBS and suspended with 500μl of binding buffer at concentration of 1×10^6 cells/ml. 5μl Annexin V-FITC (excitation/emission at λ=488nm/525nm) was added to the cell suspension and then 5μl propidium iodide (PI, excitation/emission at λ=535nm/617nm) was added. After incubated for 15 min, samples were analyzed by FacsCalibur flow cytometer (Becton Dickinson, Mountain View, CA, US), Annexin V-FITC was detected by FL1 channel, PI by FL3 channel.

Detection of mitochondrial transmembrane potential (MTP) loss by JC-1

MTP loss was assessed by JC-1 (Beyotime, excitation/ emission at λ=490nm/530nm and 525nm /590nm) following the manufacturer's instruction. Cells were stained with 1ml of staining working solution and 1ml RPMI 1640 medium. After incubated for 20 min, cells were washed with PBS three times before harvested. Ten thousand events of each sample were collected by FacsCalibur flow cytometer. Green fluorescence was detected by FL1 channel and red by FL2 channel. MTP were evaluated by the ratio of intensity of green and red fluorescence, as FL2/FL1.

Detection of ROS by DCFH-DA

2, 7-dichlorofluoroscein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA, excitation/emission at λ=488nm/525nm) was used to assess ROS following the manufacturer's instruction. After treated with emodin for a certain time, HCT116 cells were incubated with 10μmol/l of DCFH-DA for another 30 min. Ten thousand events of each sample were collected by Facs Calibur flow cytometer (FL1 channel).

Quantitative real-time reverse transcription polymerasechain reaction (qPCR)

Total RNA of 1×10^6 cells was extracted using 1ml TRIzol reagent (Invitrogen, Carlsbad, California, USA). cDNAs were synthesized with 16μl of total RNA in a 50μl reaction, using random hexanucleotide primers and Moloney murine leukemia virus (MMLV)

reverse transcriptase (Promega, Madison, Wisconsin, USA). For qRT-PCR, triplicates of 20μl PCR reactions were performed on 2μl of cDNA, using EzOmicstm SYBR qPCR kit (Biomics, Nantong, Jiangsu, China). The primers were as follows: p53: forward: 5'-CCAGGACTTCCATTTGCTTTGTC-3', reverse: 5'-CCCTACCTAGAATGTGGCTGATTG-3'; GAPDH: forward: 5'-AAGGTCGGAGTCACCGGATT-3', reverse: 5'-CTGGAAGATGGTGATGGGATT-3'. Amplification and analysis were carried out on an ABI 7500 Fast instrument. Data were analyzed using the comparative cycle threshold (Ct) method as a means of relative quantization, normalized to an endogenous reference (GAPDH) and relative to a calibrator (normalized Ct value obtained from control group cells) and expressed as $2^{-\Delta\Delta Ct}$ according to the Applied Biosystems manufacturer's protocol.

Western blot assay

Cells were resuspended in cell lysis buffer (Sangon Biotech) and then incubated on ice for 30 min to extract total proteins. Mitochondrial and cytosolic proteins were isolated using a Mitochondria/Cytosol Fractionation Kit (Sangon Biotech), according to the manufacturer's instructions. Western blots were performed as described previously (Yin et al., 2012) and detected by ChemiDOCTM XRS+ system (Bio Rad). The following antibodies were used: Anti-p53 rabbit monoclonal antibody was purchased from Epitomics Inc. (Burlingame, California, USA); Anti-Bax, -Bcl-2, -Cyt c rabbit monoclonal antibodies were purchased from Cell Signaling Technology Inc. (Boston, Massachusetts, USA); Anti-β-actin mouse monoclonal antibody was obtained from ZSGB-Bio (Beijing, China).

Small interfering RNA transfection

Small interfering RNA duplex specific to p53 (p53 siRNA) and non-specific control siRNA (NC siRNA) were provided by Beyotime. The siRNA sequences used were as follows: p53 siRNA: 5'-CUACUUCCUGAAAACAACGdtdt-3' and 5'-CGUUGUUUUCAGGAAGUAGdtdt-3'. Transfection was done using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. The final concentration of siRNA was 50nmol/l.

Statistical analysis

Results were obtained from more than three independent experiments. Statistical analysis of data was performed using one way analysis of variance (ANOVA) followed by Student's t test with GraphPad PRISM 5 (GraphPad Software Inc., San Diego, CA, USA). Error bars denoted the standard deviation (SD). The significance level was set at * $p<0.05$ and **$p<0.01$.

Results

Emodin inhibits cell viability and induces apoptosis in HCT116 cells

The inhibition of emodin on HCT116 cells growth

Emodin-Provoked Oxidative Stress Induced Apoptosis in HCT116 Cells through a p53-Mitochondrial Apoptotic Pathway
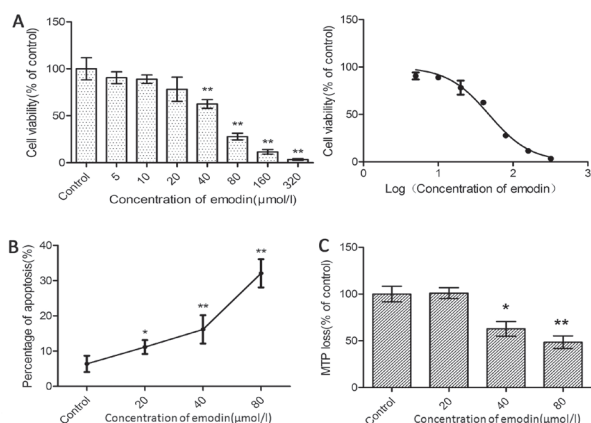

Figure 1. Emodin Induces Growth Inhabitation and Apoptosis in HCT116 Cells. A) Cell viability was determined by MTT assay after treated with emodin for 48 h (lift); transform X values using X=log(X) of A for IC50 calculation by GraphPad Prism (right). **B)** The percentage of apoptosis. Apoptosis was evaluated after treated with 20, 40 and 80μmol/l emodin for 48 h. Samples were detected by FacsCalibur flow cytometer. **C)** MTP loss was assessed by JC-1. Cells were with 20, 40 and 80μM emodin for 24h. *$p<0.05$ and **$p<0.01$ *vs* control
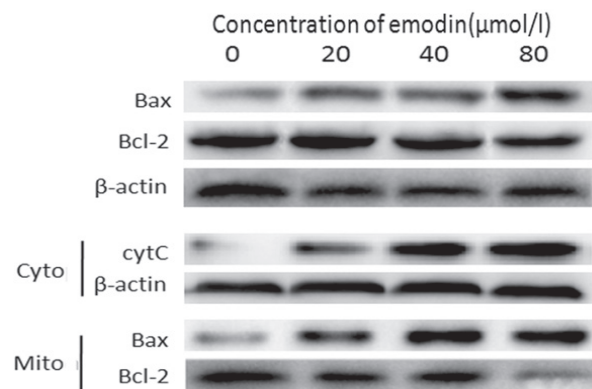

Figure 2. Expression of Mitochondrial Bax and Bcl-2 and Release of Cytochrome c. Western blot assessments were performed after treated with emodin for 24 h. Cytoplasm/mitochondria proteins were isolated using a Mitochondria/Cytosol Fractionation Kit. Data are the representative of three independent experiments

was evaluated by MTT assay. Viability of HCT116 cells exposed to emodin was reduced in a concentration-dependent manner (Figure 1. A). The IC_{50} (concentration that reduced cell viability by 50%) of emodin was 47.50±0.14 μmol/l. According to the IC_{50}, then apoptosis rates of HCT116 were examined after treated with emodin for 48 h at concentrations of 20, 40 and 80μmol/l. The appearance of increased apoptosis rates were detected by flow cytometric analysis (Figure .1B). And after incubated with emodin for 24 h, cells showed marked MTP loss in comparison to untreated control (Figure 1C), suggesting that AO-induce apoptosis in HCT116 cells maybe via a mitochondrial pathway.

Emodin affects expression of mitochondrial Bax and Bcl-2 and release of cytochrome c

To further study whether mitochondrial pathway is involved in emodin-provoked apoptosis, we evaluated the expression of mitochondrial apoptosis-related proteins. As shown in Figure 2, emodin-treatment led to increase in
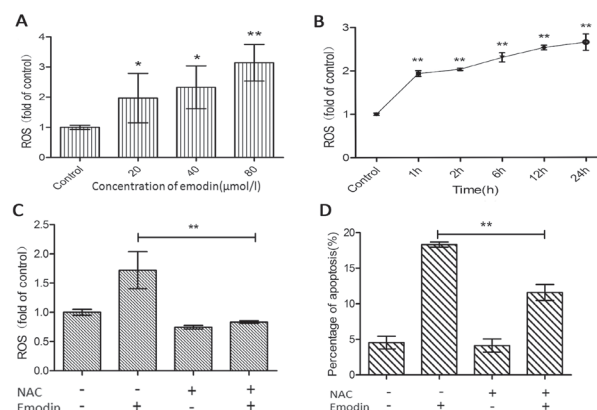

Figure 3. Emodin Induces Apoptosis via Provoking Oxidative Stress. ROS was detected by DCFH-DA on FacsCalibur flow cytometer. HCT116 cells were treated with 20, 40 and 80μmol/l emodin for 12 h **A)** or 40μmol/l for 1, 2, 6, 12, 24 h **B)**. *$p<0.05$ and **$p<0.01$ vs. control. Cells were pretreated with or without 5mmol/l NAC for 1 h. Then ROS **C)** and apoptosis (D) were examined after incubation with 40μmol/l emodin for another 12 and 48 h respectively. **$p<0.01$

Bax and decrease in Bcl-2 expression and mitochondrial translocation, and release of cytochrome c to cytosol. Taken together, all these data indicate that emodin induces HCT116 apoptosis via a mitochondria-dependent pathway.

Increase of ROS in emodin-treated HCT116 cells

We next explored the change of ROS level in the emodin-induced HCT116 cells. The results showed that emodin induced ROS increase in concentration- and time-dependent manners (Figure 3A and B). And notably, only one hour after treatment, cells displayed significantly higher ROS level than control group (Figure 3B), indicating that emodin could induce ROS increase extremely rapidly. To study the role of ROS in apoptosis, HCT116 cells were pretreated with NAC, an antioxidant to scavenge ROS, one hour before emodin to scavenge excessive ROS (Figure .3C). Emodin-induced apoptosis was attenuated in NAC-pretreated group (Figure 3D), which suggests that emodin-induced ROS could mediate apoptosis.

p53 overexpresses under oxidative stress provoked by emodin in HCT116 cells

Compared to untreated cells, emodin-treated cells exhibited markedly overexpression of p53 both at mRNA and protein level (Figure 4A). Thereafter, we silenced p53 by transfecting with p53 siRNA to study its importance in emodin-induced apoptosis (Figure 4B). In contrast to the cells transfected with NC siRNA, p53 siRNA-transfected cells exhibited decline in apoptosis rate (Figure 4C), indicating that p53 contributes to emodin-induced apoptosis in HCT116 cells. In NAC-pretreated group, p53 expression decreased compared to that treated by emodin alone (Figure 5A), suggesting that emodin-provoked oxidative stress leads to p53 overexpression.

p53 modulates Bax expression and mitochondrial translocation

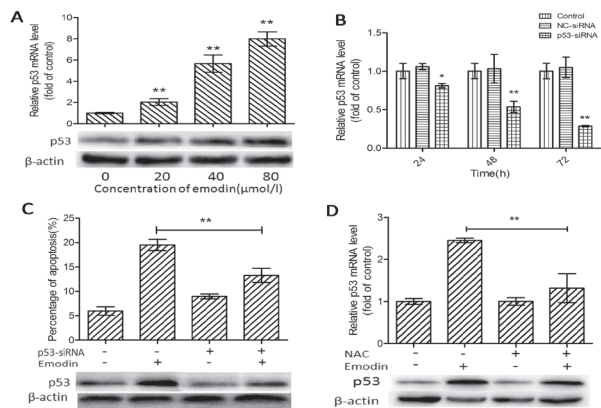To address a possible role of p53 in the emodin-

Figure 4. p53 Overexpressed Under Emodin-Provoked Oxidative Stress. A) qPCR (up) and western blot (down) analysis of p53 expression after treatment with emodin for 24 h. **B)** Silencing efficiency of siRNA detected by qPCR after cells were transfected with p53 siRNA for 24, 48 and 72 h. *p <0.05 and **p< 0.01 *vs* control. **C)** Cells were transfected with p53 siRNA for 24 h before treatment with 40μmol/l emodin, and western blots (down) and apoptosis (up) detections were performed after 24 and 48 h incubation respectively. **D)** p53 expression in mRNA and protein level after pretreated with or without 5mmol/l NAC for 1 h. qPCR (up) and western blots (down) were performed after incubation with 40μmol/l emodin for 24. **p< 0.01
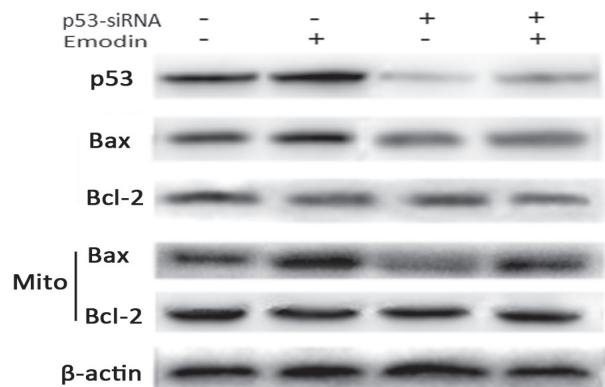

Figure 5. p53 Modulates Bax Expression and Mitochondrial Translocation. Cells were transfected with p53 siRNA for 24 h before treatment with 40μmol/l emodin, and western blots were performed after 24 h incubation. Data are the representative of three independent experiments

mediated mitochondrial apoptosis, we examined the mitochondrial translocation Bax/Bcl-2 in p53 siRNA-transfected cells. As the Figure 5 show, p53 siRNA transfection inhibits emodin-induced p53 overexpression as well as Bax expression and mitochondrial translocation, and Bcl-2 had no significant change.

Discussion

In the mitochondrial intermembrane space, there are some proapoptotic factors, like cytochrome c, Smac/Diablo, Endonuclease G and AIF, which would induce apoptosis after released into cytosol (Green et al., 1998). Activated Bax could translocate to mitochondria and oligomerize to form channels through which proapoptotic factors pass (Dejean et al., 2005). And Bcl-2 could inhibit Bax channel-forming activity (Antonsson et al., 1997). There is another channel, named mitochondrial

permeability transitionpore (mPTP), which is involved in the proapoptotic factors release. Opening of mPTP would lead to MTP collapse and matrix swelling, subsequent rupture of the outer membrane, and proapoptotic factors release into the cytosol (Petit et al., 1996; Kinnally et al., 2007). Bax/Bcl-2 also regulate the opening of mPTP as Bax promotes the the Opening of mPTP and Bcl-2 blocks it (Tsujimoto et al., 2000; Murphy et al., 2001). In our study, emodin significantly increased Bax/Bcl-2 ratios in whole cell and in mitochondria, and release of cytochrome c increased markedly in emodin-treated groups, indicating emodin could provoke apoptosis via mitochondrial pathway in HCT116 cells.

Cancer cells are known to be under increased oxidative stress presumably due to oncogenic signals activation, uncontrolled cell proliferation and dysfunction of metabolic regulation (Pelicano et al., 2004). So cancer cells are more sensitive to ROS-generating anticancer agents (Zhou et al., 2003). Our study demonstrated emodin could provoke oxidative stress in HCT116 cells. Though NAC pretreatment cannot abrogate emodin-triggered apoptosis, we still found apoptosis was reduced significantly, confirming the role of ROS in proapoptosis effect of emodin and suggesting there may be other mechanisms involved in emodin-triggered apoptosis. This result is consistent with the researches of emodin in human neuroblastoma cells, human lung cancer cells and human tongue squamous cancer cells (Su et al., 2005; Lai et al., 2009; Lin et al., 2009; Huang et al., 2013).

In anticancer researchs, overexpression of p53 is invovled in apoptosis inducted by many natrual anticancer agents (Lin et al., 2006; Hassan et al., 2013; Rengarajan et al., 2014). It has been well demonstrated that p53 could mediate transcriptional activation of Bax and trigger mitochondrial apoptosis (Toshiyuki et al., 1995; Chipuk et al., 2004). Accordingly, in our study, increase of Bax expression and mitochondrial translocation was inhibited by transfecting with p53 siRNA even though p53 siRNA cannot completely knockdown the expression of p53. Although we detected that emodin could induced decrease in Bcl-2 expression and mitochondrial translocation, transfecting p53 siRNA could not inhibit these changes, indicating there may be other factors to regulate expression and mitochondrial translocation of Bcl-2. We also found that emodin promoted p53 expression in HCT116 cells partly depending on elevation of ROS level. It should be noted that ROS elicited by emodin may not upregualte p53 expression directly. In cancer cells excessive intercellular ROS often induces oxidative DNA damage, which activates ATM and subsequently promotes p53 expression (Guo et al., 2010; Huang et al., 2011).

In conclusion, this study indeed demonstrates that emodin could trigger mitochondrial apoptosis in HCT116 cells. Our results also reveal the probable signal pathway through which emodin induced HCT116 cells apoptosis: emodin provokes ROS upregulation and subsequent p53 overexpression in response to the oxidative stress, p53 upregulate Bax expression and mitochondrial translocation, leading to apoptosis. However, ROS, as trigger of emodin-induced apoptosis is lack to be studied about the mechanism of its increase provoked by emodin.

Then further studies should unveil the generational or regulatory pathways of ROS in emodin-treated cancer cells.

Acknowledgements

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