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Full Length Research Paper

The effects of salidroside on transcription factors and cell growth inhibition in human lung adenocarcinoma epithelial cells

Yunyi Yao[1], Fan Li[2], Hua Jin[3], Yanshan Zhang[4], Jianhong Xue[2], Huijuan Wang[4], Xiaoli Zhou[5], Huarong Hong[6] and Fang Chen[1]*

[1]School of Life Sciences, Sichuan University, Chengdu 610064, P. R. China.
[2]International Medical Center, Chinese PLA General Hospital, Beijing 100853, P.R. China.
[3]2nd Department of Gynecology Oncology, Affiliated Tumor Hospital, Xinjiang Medical University, Urumqi 830011, P.R. China.
[4]Tumor Hospital of Wuwei. Wuwei, 733000, P.R. China.
[5]Sichuan Provincial School of Health, Chengdu, Sichuan, 610100, P.R. China.
[6]School of Basic Medical Science, Chengdu Medical College, Chengdu 610083, P.R. China.

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Salidroside is thought to be one of the compounds from *Rhodioladumulosa*, responsible for neuroprotective, antidepressant, anxiolytic and anti-tumor actions. Our research focused on the effects of Salidroside on transcription factors and cell cycle inhibitors in A549 cells. We found out that the gene and protein expression of c-Myc were down-regulated significantly, while it was the opposite with P21. C-fos is slightly down-regulated mainly on mRNA level while P27 is slightly up-regulated both on mRNA level and protein level. No significant changes have been detected on c-jun and CEBP-α. This suggested that c-Myc and P21 may play a key role in the pharmacological action of Salidroside. Also, we found that the growth of A549 cells was inhibited and the cell cycle was arrested at G0/G1 phase. P21 plays an important role in cell growth inhibition via cell cycle arrest.

Key words: Salidroside, adenocarcinoma epithelial cells, c-Myc, P21, cell cycle, cell viability.

INTRODUCTION

Rhodioladumulosa, widely distributed at high altitudes in the arctic and mountainous regions throughout Asia and Europe, is a perennial herbaceous plant with various medical uses. Salidroside ($C_{14}H_{20}O_7$), with the IUPAC name of 2-(4-hydroxyphenyl) ethyl β-D-glucopyranoside, is one of the main active constituents from *Rhodioladumulosa*. Salidroside has been used as one of the compounds responsible for neuroprotective, antidepressant and anxiolytic actions (Yu et al., 2008; Perfumi et al., 2007; Li et al., 2007). Recently, there are some researches of Salidroside on the anti-tumor effect (Skopińska-Rózewska et al., 2008). However, the mechanism of pharmacological effect of Salidroside is still unclear. According to former studies, all of these pharmacological actions have a certain relationships with transcription factors related with cell cycle and cell growth inhibition (Wahle et al., 2010). A better understanding on these molecular factors in the pharmacological action of Salidroside will help us to know more about this natural product and may help us to have a new insight on the medical treatment of Salidroside. Meanwhile, the cell cycle and cytotoxicity had also been detected in our research. All these could help us to well know the effects of Salidroside on cancer cell inhibition both on cell and molecular level. This is also one of the potential drugs to treat various tumors including human lung cancers.

*Corresponding author. E-mail: fangchen151@yahoo.com.cn.

Table 1. Primers of each gene for RT-PCR analysis.

Gene	Up primer 5'-3'	Down primer 5'-3'	Length of product (bp)	Tm (°C)
c-myc	ACTATGACCTC GACTACGACTC	CAGATCCTGCA GGTACAAGCTG	512	57
c-fos	CTGGCGTTGTG AAGACCATGAC	GCTCAGGGTCA TTGAGGAGAG	420	59
c-jun	TCAGCAACTT CAACCCAGGC	TGTTTAAGCT GTGCCACCTG	412	56
CEBP-α	TCCATCGACAT CAGCGCCTAC	CAGCTGCTTGG CTTCATCCTC	339	61
p21	TCAGAACCCAT GCGGCAGCAAG	TGGAGTGGTAG AAATCTGTC	434	55
p27	AAGCACTGCAG AGACATGGAAG	GTCTTCTGAGGC CAGGCTTCTTG	448	60
β-actin	CTCCATCCTG GCCTCGCTGT	GCTGTCACCT TCACCGTTCC	268	55

MATERIALS AND METHODS

Cell culture and drug treatments

Human lung epithelial cells A549 were propagated at 37°C with 5% CO_2 in RPMI 1640 medium (Invitrogen) supplemented with L-glutamine, 100 units/ml *penicillin*, 100 µg/ml *streptomycin*, and 10% (v/v) fetal bovine serum (FBS). Salidroside was purified by HPLC and then dissolved in 0.9% normal sodium (NS) and stored at 4°C. The concentration of 20.0 µg/ml will get a good result on the pharmacological action of Salidroside according to previous researches.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

As regards the total RNA of A549 cells, both the treated cells with Salidroside at the concentration of 20.0 µg/ml for 12 h and the control groups were isolated from A549 cells. Oligonucleotide primers of each gene (Table 1) were synthesized by Takara Company based on the sequences of human genes searching from NCBI. After reverse transcription, all the products were used as the templates for the polymerase chain reaction (PCR). Hot start (94°C for 1 min) PCR amplifications were carried out on a PCR machine (PE 9600) for 30 cycles with each cycle consisting of a denaturing step, an annealing step and an elongation step followed by a final extension step of 10 min at 72°C. 10 µl of each PCR product was detected for the gene expression.

Western blot analysis

A549 cells were treated with Salidroside at a concentration of 20.0 µg/ml for 36 h and then harvested together with the control groups. Total protein extracted (20 µg) was boiled at 100°C with loading buffer for 5 min, and then subjected to 12.5% SDS-PAGE. After electrophoresis, gels were briefly equilibrated in transfer buffer then transfered onto nitrocellullose membranes. Transfer was performed at 70 V for 2 h at 4°C. After blocking in 5% Nonfat milk for 1 h, membranes were incubated overnight at 4°C with primary antibody (rabbit polyclonal IgG, Millipore) in 0.5% blocking solution. After washing with PBS, the membranes were incubated with secondary antibody (Goat Anti-Rabbit IgG, QIAGEN) for 1 h at room temperature, washed again with 1X PBS(pH 7.4); photos were taken with film exposures for analysis.

3, 5-dimethylthiazol-2-yl)-2, 5-dephenyltetrazolium bromide (MTT) assay for cytotoxicity detection

Viability of A549 cells was determined by MTT assay after treatment of Salidroside at a concentration of 20.0 µg/ml. 8×10^3 cells/well were seeded into 96-well plates. Salidroside was diluted in DMEM/10% fetal bovine serum. These diluted materials were separately incubated with A549 cells for 24 to 72 h. Triplicate measurements were made for each time point. At the end of the treatment, 20 µl of 2 mg/ml MTT (3, 5-dimethylthiazol-2-yl)-2, 5-dephenyltetrazolium bromide, Sigma, USA) was added into each well, and the plates incubated for 2 h at 37°C. 100 µl acidic isopropanol was added into each well, then optical absorbance at 595 nm was measured using a scanning multiwell spectrophotometer plate reader.

Cell cycle analysis by flow cytometry

A549 cells after treatment of Salidroside at a concentration of 20.0 µg/ml for 24 to 72 h were seeded in 6-well plates by a density of 1×10^5 /ml. Cells were trypsinized, centrifuged (1000 r/min, 5 min), washed with 1X PBS, fixed with cold 70% ethanol/30% phosphate-buffered saline at 4°C overnight. Cells were digested by

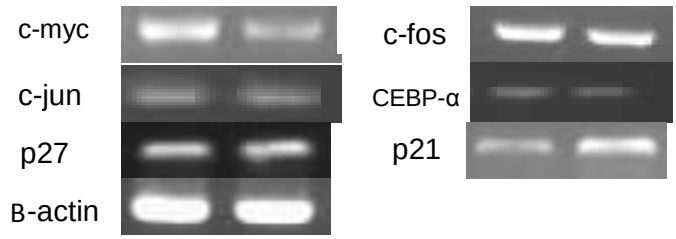

Figure 1. Detection of gene expression in A549 cells. Left line is control group while right line is the group treated with Salidroside (20.0 μg/ml for 12 h).

1000 U RNase A (Takara), and then stained with 1% propidium iodide at 37°C for 30 min. The DNA profiles were determined within 4 h of staining by flow cytometry (FCM, PARTEC).

Statistical analysis

All values were expressed as mean±SD. The results were evaluated by one-way ANOVA and Tukey's multiple comparison tests. Statistically significant differences between groups were defined as $p < 0.05$. Calculations were performed with SPSS 12.0 software (SPSS, Chicago, USA).

RESULTS

Effects of Salidroside on the gene expression

As shown in Figure 1, c-myc was down-regulated at the gene level after treatment of Salidroside. The expression of CEBP-α was slightly decreased while the expression of p21 increased significantly after treatment of Salidroside in A549 cells. No obvious changes had been detected on c-fos, c-jun and p27. All data were analyzed in Figure 2.

Effects of Salidroside on the protein expression

As shown in Figure 3, the expression of c-Myc at the protein level was down-regulated after treatment with Salidroside, just the same with the change of gene expression. However, increased expression of P21 was detected in A549 cells, together with the change of gene expression. C-fos was slightly down-regulated while no obvious change on P27 was detected. All data were analyzed in Figure 4.

Effects of Salidroside on cell viability

To determine the effects of cell viability and inhibition of Salidroside, the viability of the treated and control group of cells were measured by MTT assay. As shown in Figure 5, Salidroside did show significant cytotoxic effect and cell growth inhibition on A549 cells. Compared with control group, the viability of A549 cells treated with Salidroside for 24 to 72 h was significantly inhibited at concentration of 20 μg/ml.

Analysis on cell cycle

As shown in Table 2, Salidroside could induce G_0/G_1 phase cell cycle arrest in A549 cells. Compared with control group, the percentage of cells at G_0/G_1 phase was increased significantly, while the percentage of S phase decreased significantly, compared with the control group; G_2 / M phase cells also decreased. All these could indicate that Salidroside inhibited the cell growth at G_0/G_1 phase.

DISCUSSION

Salidroside is one of the main active constituent compounds from *Rhodioladumulosa* responsible for the neuroprotective, antidepressant and anxiolytic actions and anti-tumor (Yu et al., 2008; Perfumi et al., 2007; Li et al., 2007; Skopińska-Rózewska et al., 2008). Here, we used Human lung adenocarcinoma epithelial cells A549 to detect the expression change of these transcriptional factors involved in the cell cycle under the treatment of Salidroside, both on gene and protein level. Our research has found out that Salidroside could inhibit the gene and protein expression of c-Myc obviously in A549 cells, while it is the opposite with P21. c-fos, c-jun and CEBP-α are slightly down-regulated mainly on gene level and P27 is slightly up-regulated both on mRNA level and protein level.

Transcriptional factors bind to specific DNA sequences and thereby control the transcription of genetic information and adjust the cell function. c-myc is the most frequently deregulated oncogene in many human cancers including both the lung small cell cancer and lung non small cell cancer (Powers and Mu, 2008; Danesi et al., 2007). This factor plays an important role in inflammation, oxidative stress, cell growth, apoptosis, differentiation (El-Bassiouni et al., 2006; Carroll et al., 2002; Couillard et al., 2009). Here, we showed that the gene expression of c-myc had been down-regulated after 12 h of Salidroside treatment in A549 cells. It is the same at protein level after 36 h of Salidroside treatment in A549 cells. So we consider that Salidroside induces its pharmacological actions by adjusting the transcription factor c-myc.

C-fos is also one of the transcriptional factors playing roles in cell proliferation, apoptosis, differentiation, and transformation (Gomard et al., 2008; Mahner et al., 2008). C-Jun, a major constituent of AP-1 transcription factor transducing multiple mitogen growth signals, is frequently overexpressed in non-small cell lung cancers (NSCLCs) (Reddy and Mossman, 2002; Heasley et al., 2008). CEBP-α is a bZIP transcription factor which can bind as a homodimer NtoN certain Npromoters and enhancers. The encoded protein has been shown to bind

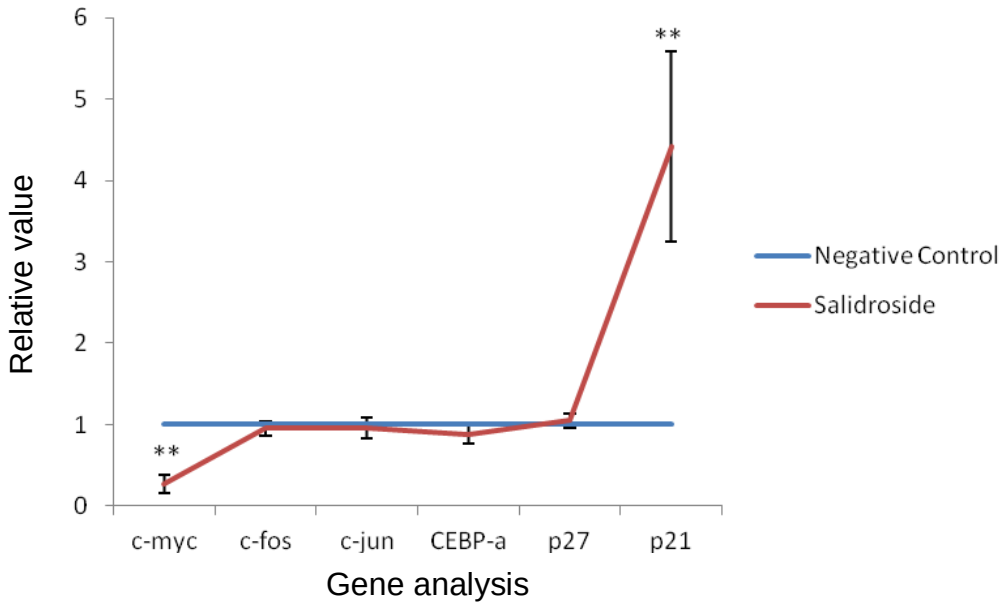

Figure 2. Analysis for gene expression. **, p<0.01 compared with negative control.

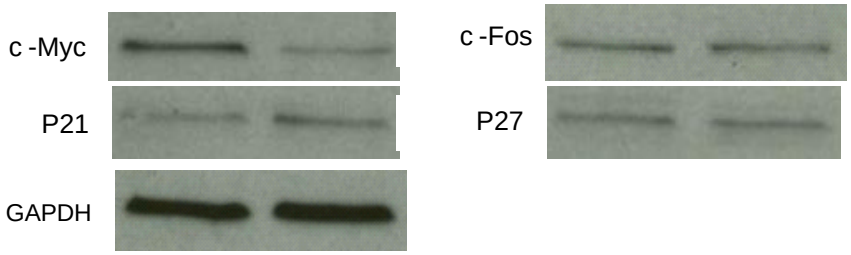

Figure 3. Detection of protein expression in A549 cells. Left line is control group while right line is the group treated with Salidroside (20.0 µg/ml for 36 h).

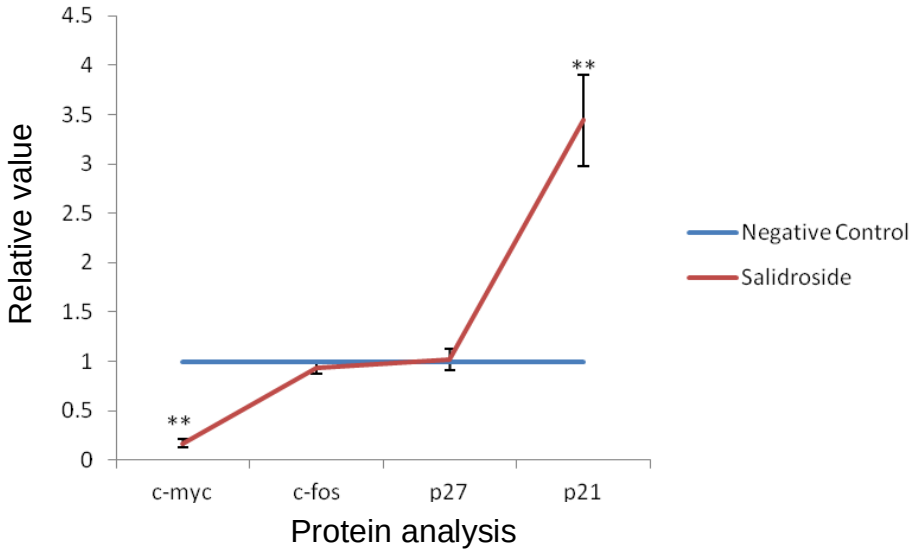

Figure 4. Analysis for protein expression. **, p<0.01 compared with negative control.

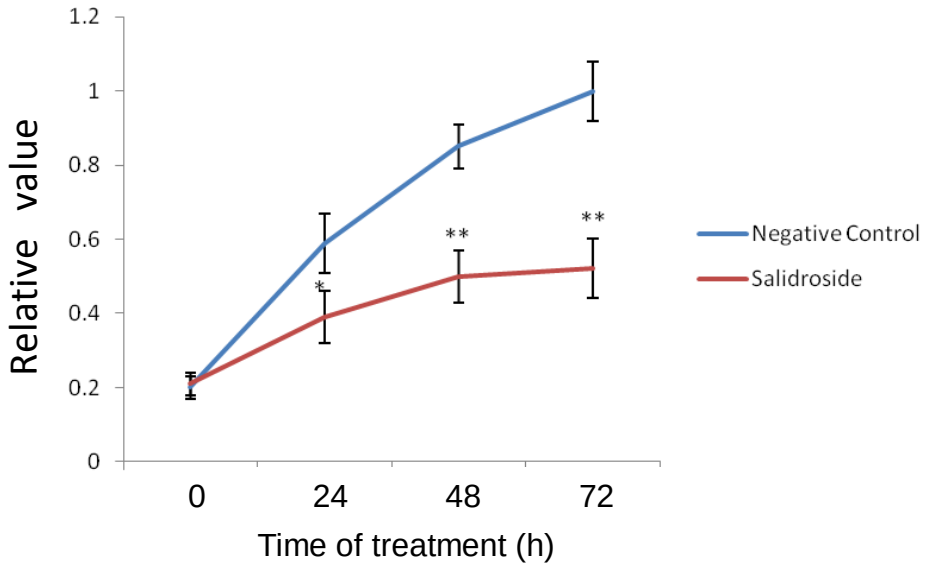

Figure 5. Cell viability test by MTT. *, p<0.05, ** p<0.01 compared with negative control.

Table 2. Cell cycle analysis by FCM.

Time of treatment (h)	G0/ G1 (%)	S (%)	G2/M (%)
0 (NC)	50.8+2.2	33.5+1.8	15.6+0.8
12	62.7+2.5**	22.7+1.6**	14.6+0.9
24	65.2+2.7**	23.7+1.7**	11.1+1.1**
36	66.2+2.8**	22.4+1.7**	11.4+1.2**

Compared with 0 h (Negative control), **, p<0.01.

to the promoter and modulate the expression of the gene encoding leptin (Gregoire, 2004), also it can inhibit cell cycle kinases and cause growth arrest in cultured cells (Koschmieder et al., 2009). Here, we found out the gene expression CEBP-α decreased slightly after Salidroside treatment while without significant change. No obvious changes on c-jun and c-fos had been detected. For protein expression, c-fos decreased slightly after Salidroside treatment while without significant change.

Former researches had indicated that Salidroside can adjust the growth, apoptosis, and differentiation of many cells, such as neural cells, myocardial cell, some cancer cells and oxygen deficiency condition cells. Consider that cell cycle inhibitors, especially P21 and P27 play a very important role in these processes (Lu et al., 2010), we detected the expression of P21 and P27. P21, a potent cyclin-dependent kinase inhibitor (CKI), binds to and inhibits the activity of cyclin E/CDK2 complexes, and thus functions as a regulator of cell cycle progression at G_0/G_1, which result in cell cycle G_0/G_1 arrest (Bae et al., 2001; Chattopadhyay et al., 2007). P21 can also be involved in the growth, apoptosis, and differentiation of many cells (Ukomadu et al., 2003; Wang et al., 2008). In normal A549 cells, the expression of P21 is low both on the gene and protein level (Ogretmen et al., 2001). However, it increased both on gene level and protein level after the treatment of Salidroside. This suggests that P21 should take part in and act as a critical cell cycle inhibitor in the cell cycle arrest, the cell growth, apoptosis, and differentiation in the pharmacological actions of Salidroside. P27 is another cyclin-dependent kinase inhibitor, which binds to and prevents the activation of cyclin E/CDK2 or cyclin D/CDK4 complexes, and thus controls the cell cycle progression at G_0/G_1 (Dobashi, 2005). No obvious changes on P27 had been detected on gene and protein level after the treatment of Salidroside.

Cell cycle analysis is a useful method to distinguish cells in different phases of the cell cycle using FCM. Cell cycle anomalies can be symptoms for various kinds of cell damage, indicating the abnormal cell growth and inhibition (He et al., 2011). The MTT assay is a colorimetric assay for measuring the viability and the proliferation of cells. It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit

cell viability and growth. The results indicated that A549 cells were arrested at G_0/G_1 phase and thus the growth was inhibited. These mean that Salidroside has some cytotoxicity on human lung cancer cells.

This research mainly focused on the effects of Salidroside on the cell cycle and cell growth of human adenocarcinoma epithelial cell A549. Rather than other TFs, c-myc and p21 were involved obviously in the treatment. c-myc was down-regulated while p21 was up-regulated significantly, both on gene and protein level. The growth of A549 was inhibited after treatment with Salidroside at a concentration of 20.0 µg/ml and the cell cycle was arrested at G0/G1 phase.

This research will help us to better understand the molecular mechanism of Salidroside on the anti-tumor effects. Considering its low toxicity, Salidroside may be the potential drug for further cancer treatment.

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