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20 (S)-Ginsenoside-Rh2 Induces Apoptosis in Human Lung Adenocarcinoma A549 cells by Activating NF-κB Signaling Pathway

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Inducing apoptosis of tumor cell is one of the main therapeutic measures on tumor. 20 (S)-Ginsenoside-Rh2 (Rh2) is a kind of antineoplastic agents developed from plants. In this study, the effect of Rh2 on apoptosis and its underlying mechanisms were investigated in human lung adenocarcinoma A549 cells, using the methylthiazoletetrazolium (MTT) assay to detect cell survival, Hoechst33258 staining to observe morphological changes during apoptosis through inverted fluorescence microscope, dual luciferase reporter system to detect NF-êB-Luc relative luciferase activity, immunofluorescence assay to test phosphorylation expression level and nuclear translocation of transcription factor NF-αB p65, RT-PCR to analyse TNF-α mRNA expression level. The results showed that Rh2 could inhibit cell proliferation and induce apoptosis in A549 cells. Compared to the control group, relative luciferase activity of NF-κB-Luc reportor gene significantly improved in A549 cells treated with Rh2, nuclear NF-κB p65 fluorescence luminance increased obviously, and the expression of TNF-α mRNA remarkably enhanced. Taken together, these results suggest that Rh2 can induce apoptosis of A549 cells by activating NF-êB signaling pathway and up-regulate the expression of TNF-α.

Key words: 20 (S)-Ginsenoside-Rh_2, human lung adenocarcinoma A549 cells, Apoptosis, NF-Kb, TNF-α.

Lun[1]g cancer is a major death cause of malignant tumor victims in the world. Human lung adenocarcinoma is a common type of lung cancer, which has high toxicity and transfers quickly(Liberman *et al.,* 2006). The development of tumors is a complicated process of multiple factors involved, multiple gene changes, multi-stage, many steps of evolution, and its pathogeny and etiopathogenesis have not yet been fully clarified(Zeng *et al.,* 2003). The occurrence of cancers is related to not only the abnormal cell proliferation, but also the abnormal cell apoptosis from cytology point of view. The research of NF-êB family and NF-κB-mediated signal transduction pathways in cell apoptosis is becoming the domestic and foreign hot spot. Recent studies have shown that the activation of NF-κB can promote cell apoptosis(Piotrowska *et al.,* 2008; Sethi *et al.,* 2007).

Cell apoptosis or programmed cell death is an active death process of cell controlled by gene. Inducing apoptosis of tumor cells is an important anti-tumor mechanism(Tsuruo *et al.,*

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2003). At present most chemotherapeu- tics can induce apoptosis of tumor cell, but side effects limit its clinical application, so looking for safe and effective anti-tumor drugs has attracted more and more people's attention.

20 (S)-Ginsenoside-Rh2(Fig. 1), the molecular formula $C_{36}H_{62}O_8$, whose chemical name is 20 (S)-protopanaxadiol- 3-O-beta-D-glucopyranoside, is a kind of natural active substance known and existing in ginseng, belongs to panaxadiol saponins, and has strong anti-tumor pharmacological action(Ham *et al.,* 2006; Ha *et al.,* 2010). The study found that anti-tumor activity of Rh2 is related to blocking the cell cycle, Rh2 can regulate G1-phase cell cycle regulatory protein, induce cell growth arrested at G1-phase, which will then result in apoptosis(Yim *et al.,* 2003; Ham *et al.,* 2003;Choi *et al.,* 2009). To induce apoptosis of tumor cells through many paths is one of the most important mechanism of Rh2 anti-tumor activity. Cheng CC et al.(2005) found that up-regulation of DR4 death receptor played a key role in Rh2 inducting apoptosis of human lung adenocarcinoma A549 cells, ultimately apoptosis occurred through extrinsic apoptosis pathway of Caspase-8/Caspase-3. Zhou DB et al. (Zhou *et al.,* 2005). reported that Rh2 could induce apoptosis of lung adenocarcinoma A549/DDP with Fas/FasL system approach by up-regulation of p53 and Fas, as well as down regulation of expression of Bcl-2. However, signal transduction pathways and targets of Rh2 anti-lung cancer effect are still unclear. In this study, the aim was to investigate whether or not Rh2 could induce cell apoptosis and preliminarily explore its relative molecular mechanisms in human lung adenocarcinoma A549 cells, and provide experimental basis for further development and application.

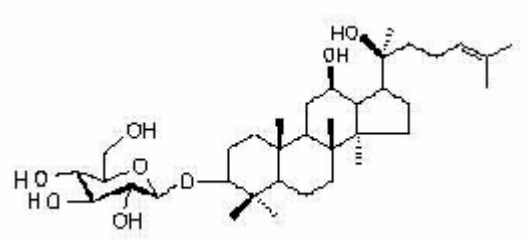

Fig. 1. Chemical structure of 20 (S)-Ginsenoside-Rh2

MATERIALS AND METHODS

Cell culture and reagents

Cell culture: Human lung adenocarcinoma cell line A549 cells were obtained from Cell Culture Center for Peking Union Medical College (Beijing, China). The cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37° in an atmosphere containing 5% CO2. Cells at logarithmic growth phase were used for the experiments.

Reagents

20 (S)-Ginsenoside-Rh2 was purchased from China Institute of Drug and Biological Products. Reporter plasmid NF-êB-luc was presented by Chinese National Human Genome Center. Fetal calf serum was purchased from Hyclone (USA), DMEM was purchased from Gibco company (Gibco/Invitrogen Corp, USA).

Cell proliferation analysis

Suspended cells were seeded in 96-well cell culture plate (100 µl/well) and incubated for 24h, synchronization with 0.1% fetal calf serum for 24h, then the cells were treated with various concentrations of Rh2 for 24h, 48h or 72h, 20 µl MTT (5 g/L, Sigma, USA) was added and cells were cultured for additional 4h. Subsequently, cells were lysed using dimethylsulfoxide (150 µl/well, Pierce Biotechnology, USA). When the formanzan crystals were completely dissolved, and the A was determined using a Bio-Rad microtiter reader at A570.

The 50% inhibitory concentration (IC50) was determined as the drug concentration causing 50% reduction in cell viability. The degree of resistance was calculated by dividing the IC50 for A549 cells. The fold-reversal was calculated by dividing the IC50 for cells. The inhibitory ratio was calculated by the following formula: (1-A570 value of experimental group/A570 value of control group)×100%, then using SPSS 10.10 to calculate IC50 by linear regression method.

Apoptosis identified by Hoechst33258 staining

A549 cells were treated with the presence or absence of Rh2 for 24h, after washed three times with PBS and fixed with 10% paraformaldehyde for 10min. Afterward the fixed cells were washed and labeled with Hoechst 33258 (10 µg/mL, Sigma, USA) at room temperature in the dark for 10 minutes. After washed three times with PBS and observed

using fluorescence microscopy with standard excitation filters, apoptotic cells stained brightly and displayed condensed and fragmented nuclei, normal cells showed an even distribution of the stain throughout the nucleus with flocculated chromatin. Then 500 cells were counted with their nuclear fragmentation visualized under a fluorescence microscope, the percentage of apoptotic nucleus was calculated by the following formula: apoptotic nucleus/500×100%, all cells from three random microscopic fields at 400× magnification were counted.

Cell transfection and Luciferase assay

Proceeded transfection according to the instructions of eukaryon transfection kit (Vigorous, Beijing). 24h after transfection, cells were stimulated for 24h with Rh2 or DMSO, then transfected cells were harvested and lysed using the lysis buffer. The cell was used to measure luciferase activity using the dual-luciferase reporter (DLR) assay kit (Promega, USA). The firefly luciferase activity was normalized to the Renilla luciferase activity. All experiments were carried out in triplicate, and the data are presented as the mean luciferase $x^- \pm$ SD (n=3). NF-κB activity of the control group as 1 (100%), calculated the relative NF-κB luciferase activity of other groups.

Immunofluorescence

A549 cells were washed in phosphate-buffered saline (pH 7.4) which contained 0.1% TritonX-100 and fixed in methanol for 10 min, washed three times in PBS for 5 min each time. The cells were then incubated with a phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb (Cell signaling, USA) at 1:200 dilution at 4! overnight. Then the cells were washed three times in PBS for 5 min each time. Following by the incubation of Cy3-conjugated anti-rabbit IgG secondary antibody (Cell signaling, USA) at 1:200 dilution for 45 min at room temperature. After washing with PBS three times, then stained with DAPI (1: 500 dilution) for 5 min at room temperature. After washing, the cells were mounted and the fluorescent images were collected using a fluorescence microscope (Olympus IX81). Each experiment was repeated at least three times.

RT-PCR analysis

After cells were treated with 20 μg/mL Rh2 for 24h, total RNA was extracted from the cultured cells using Trizol reagent (Invitrogen) according to the manufacturers protocol. The cDNA was synthesized by extracting and reverse transcribing the total cellular RNA according to the manufacturer's instructions. The β-actin gene was selected as the most stably expressed control gene for normalization. PCR was performed with primers for TNF-β [52 -CAG GCA GTC AGATCATCTTC-32 (sense); 52 -CTTGATGGC AGA GGA GGT-32 (antisense)] and â-actin [52 -AAGGATTCCTATGTGGGC-32 (sense); 52 -CATCTCTTGCTCGAAGTC-32 (antisense)]. RT – PCR was detected using a one-step RT-PCR kit (Takara Biotechnology) according to the manufacturer's instructions. The cycling conditions: 94°, 5min, followed by 35 cycles of 94°, 50s; 54°, 50s; 72°, 50s, and a final extension of 72° for 10 min. PCR products were separated on the 1.5 % agarose gel viewed by ethidium bromide (EB) staining. These data were acquired with Alpha Imager 2200 software.

Statistical analysis

The differences were tested using ANOVA. All values are expressed as $x^- \pm$ S.D., and statistical significance was defined as $p<0.05$

RESULTS

The inhibitory effect of Rh2 on A549 cell proliferation

To detect A549 cell proliferation ability by MTT analysis. The results showed that compared with the control group, the negative control group of cosolvent DMSO hadn't obvious changes ($p>0.05$). However, addition of Rh2 inhibited cell proliferation ability, A570 value obviously declined ($p<0.05$), and the inhibitory effect was in a dose-dependent manner . After A549 cells were treated with 25 μg/mL Rh2 among these, the inhibitory ratio was 33.6%, and IC50 was 28.5 μg/mL (Fig. 2). These results demonstrated that Rh2 could affect A549 cell proliferation ability, and inhibit their growth.

Effect of Rh2 on A549 cell apoptosis

Hoechst33258 staining revealed that in the control group A549 cell nucleus had regular contours and were round or oval, and the fluorescences distributed well. However, typical morphological change of apoptotic cells were observed after A549 cells were treated with 30 μg/mL Rh2 for 24h, which showed characteristic of

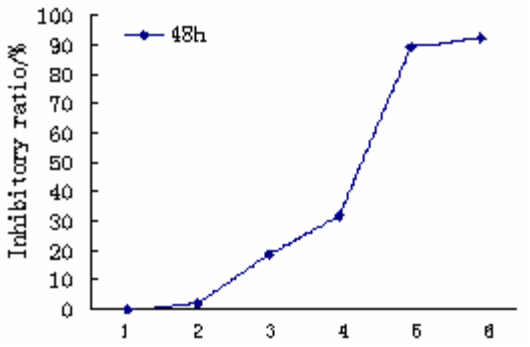

Fig. 2. The proliferation inhibition effect of Rh2 on A549 cells. 1 (Ctrl): Cells under nomal condition; 2 (DMSO): Cells and cosolvent DMSO; 3 (12.5 μg/mL): Cells treated with 12.5 μg/mL Rh2; 4 (25 μg/mL): Cells treated with 25 μg/mL Rh2; 5 (50 μg/mL): Cells treated with 50 μg/mL Rh2; 6 (100 μg/mL): Cells treated with 100 μg/mL Rh2 Values are given as mean ± S.D. from 3 independent experiments in triplicate and $p<0.05$ is considered statistically significant. *$p>0.05$, #$p<0.05$, ##$p<0.01$ vs Ctrl

chromatin condensation, nuclear fragmentation, uneven distribution of fluorescences and the enhancement of luminance, the chromatin divided into blocks and particles, and the appearance of apoptotic bodies of luminance enhancement (Fig. 3a). Statistical analysis showed that the percentage of apoptotic nucleus in the control group was 19.562±4.685, however, it was 48.846±5.973 after A549 cells were treated with 30 μg/mL Rh2, and there was significant difference between the two groups ($p<0.01$) (Fig. 3b). The results proved that Rh2 could induce A549 cell apoptosis.

Effect of Rh2 on NF-êB transcription activity

After luciferase reporter plasmid, Renilla luciferase plasmid, and green fluorescence protein plasmid were extracted, we verified them using 1% agarose gel electrophoresis. Then transfection was proceeded, the effect of Rh2 on NF-êB transcription activity was analysised by analysising luciferase expression. The results showed that NF-êB-Luc Relative luciferase activity was (100%±3.21)% in

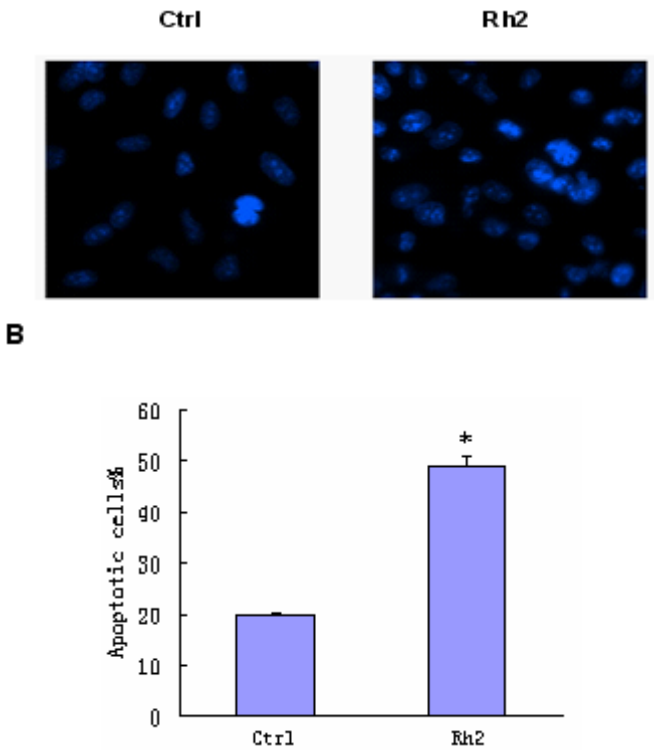

Fig. 3. Fluorescence microscopy evaluation of A549 cells apoptosis by Hoechst33258 staining. (A) Effect of Rh2 on apoptotic nuclear morphological in A549 cells for 24h (Hoechst33258×400). (B) the percentage of apoptotic nucleus. Ctrl: Cells under normal condition; Rh2: Cells treated with 30 μg/mL Rh2. *$p<0.05$ vs Ctrl.

control group, was (107±2.35)% in DMSO negative control group, and was (269±4.51)% in 20 μg/mL Rh2 group (Fig. 4). These results demonstrated that Rh2 could activate transcription factor NF-κB.

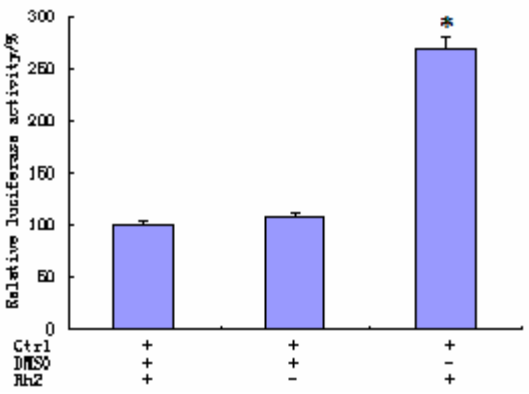

Fig. 4. Relative luciferase activity analysis of NF-κB-Luc with Rh2 in A549 cells for 24h. Each data represents the average of three independent experiments.Ctrl: Cells under nomal condition; DMSO: Cells and cosolvent DMSO; Rh2:Cells treated with 20 μg/mL Rh2. *p<0.01 vs Control

Rh2 increases NF-κB phosphorylation expression and nuclear translocation

In order to further confirm the adjustment effect of Rh2 on NF-êB signal transduction pathway, we conducted positioning, characterization, quantitative analysis of NF-κB phosphorylation expression level and nuclear translocation using immunofluorescence assay. The experimental results showed that the fluorescence luminance of nucleus marked by NF-κBp65 increased significantly, when 20 μg/mL Rh2 treated A549 cells for 0.5h-1h (Fig. 5a). Red fluorescence luminance values were analysised using Imageproanalyzer 6.3 software, the fluorescence luminance value was 71.53882 in control group, however, was 103.7358 in Rh2 group, the fluorescence luminance obviously increased(*p<0.05) (Fig. 5b), which indicated that Rh2 could improve NF-êB phosphorylation expression and the rate of entering into nucleus.

Effect of Rh2 on TNF-α mRNA expression

In order to further confirm the mechanism of Rh2 inducing cell apoptosis, which may be that Rh2 activate NF-αB, and then regulate target gene expression as well as induce cell apoptosis, so we detected TNF-α mRNA expression, it is cytokine

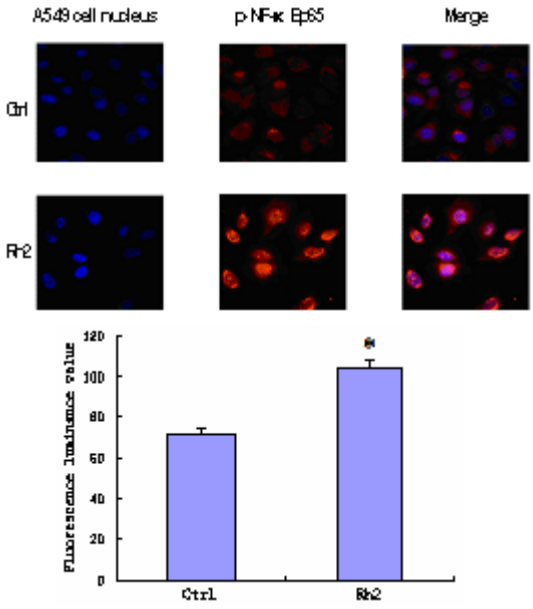

Fig. 5. Effect of Rh2 on p-NF-κBp65 by immunofluorescence. (A) the analysis of quality and orientation for Effect of Rh2 on p-NF-κBp65 for 0.5h.(B) quantitative analysis for fluorescence luminance. Ctrl: Cells under nomal condition; Rh2: Cells treated with 20 μg/mL Rh2. *p<0.05 vs Ctrl.

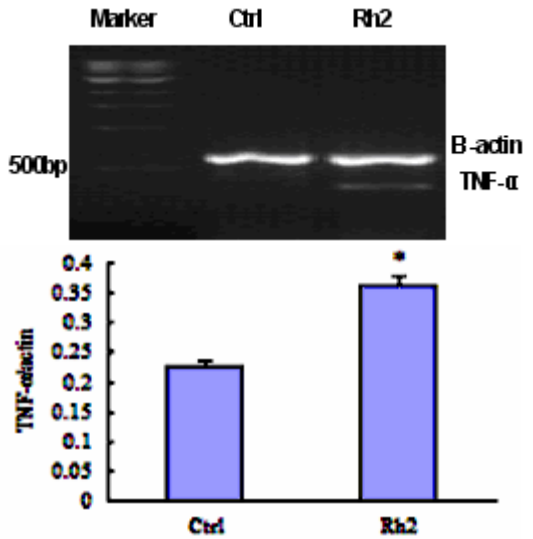

Fig. 6. Detect the expression of TNF-α mRNA treated with Rh2 in A549 cells by RT-PCR. (A) total RNA was extracted and TNF-α mRNA was amplified by RT-PCR and detected by agarose gel electrophoresis. (B) Densitometry graphs showing relative changes in mRNA expression normalized to β-actin. Ctrl: Cells under nomal condition; Rh2: Cells treated with 20 μg/mL Rh2; *p<0.05 vs Ctrl.

related to apoptosis. The result showed that compared with the control group, 20 μg/mL Rh2 increased TNF-α mRNA expression ($p<0.05$). Therefore, Rh2 could up-regulate TNF-α expression (Fig. 6).

DISCUSSION

The genesis and progression of tumor is a complex process, in a sense, the occurrence of cancers reflects a failure in the balance between cell proliferation and apoptosis in tumor, excessive proliferation and/or inhibition of apoptosis play an important part in the pathogenesis of cancer, so inducing apoptosis of tumor cell may be one of effective means to cure tumor. Rh2 has the effect of non-organ specificity abroad spectrum anti-tumor. In the study on SMMC-7721 cells, researchers found that Rh2 could effectively lower telomerase activity and transcription by influencing human telomerase reverse transcriptase, as well as to induce cell differentiation. And up-regulate the expression of P21 and P16, down-regulate D1 and cyclin E activity, arrest cell at G1/S period, eventually cause cell apoptosis(Zeng *et al.,*2003). Recent report said that Rh2-induced apoptosis was accompanied by the down-regulation of antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1. It also caused induction of the proapoptotic members Bak, Bax, and Bim leading to mitochondrial translocation of Bax and activation of caspases 9/3(Kim *et al.,*2003). A recent study found that Rh2 induced internalization of rafts and caveolae, leading to Akt inactivation, and ultimately apoptosis. Because elevated levels of membrane rafts and caveolae, and Akt activation have been correlated with cancer development, internalization of these microdomains by Rh2 could potentially be used as an anti-cancer therapy(Park *et al.,*2010). However, Rh2 is related to the study on inducing cell apoptosis and the discussion on its mechanism in human lung adenocarcinoma A549 cells, which was little reported.

In the preceding work of our project group, we found that Rh2 could inhibit cell proliferation by triggering G1 phase arrested and induce cell apoptosis in human lung adenocarcinoma A549 cells(Zhang *et al.,*2011). The cell proliferation capacity was evaluated using MTT analysis. The results showed Rh2 reduced A549 cell proliferation ability in a dose-dependent manner. After A549 cells were treated by 20 μg/mL Rh2 for 48h, the cell proliferation inhibitory ratio was 33.6%, IC50 value was 28.5 μg/mL. The above results indicated that Rh2 could affect A549 cell proliferation capacity, inhibit their growth. If cell proliferation ability decreased, could cell apoptosis occur? In order to confirm this question, we further identified cell apoptosis using Hoechst33258 fluorescent staining in morphological terms. The results showed that Rh2 could induce A549 cells morphological changes, and the morphologic characteristics of cell apoptosis emerged, such as nuclear condensation, the inconformity of the fluorescence distribution and luminance enhancement, the division of chromatin into blocks and particles, in addition, apoptotic bodies of luminance enhancement were also seen, and compared with control group, the percentage of apoptotic nucleus was enhanced significantly.

NF-κB (nuclear factor-kappaB) is a collective name for inducible dimeric transcription factors composed of members of the Rel family of DNA-binding proteins that recognize a common sequence motif. NF-κB is normally sequestered in the cytoplasm of nonstimulated cells and consequently must be translocated into the nucleus to function. The subcellular location of NF-êB is controlled by a family of inhibitory proteins, IêBs, which bind NF-êB and mask its nuclear localization signal, thereby preventing nuclear uptake. Exposure of cells to a variety of extracellular stimuli leads to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of IêB, which frees NF-κB to translocate to the nucleus where it regulates gene transcription(Karin *et al.,*2000). NF-êB participates in lots of important physiological and pathologic process, affects nearly every link of the immune system development, response and inflammatory reaction(Karin *et al.,*2002). Research shows that NF-êB family and NF-êB-mediated signal transduction pathways correlate closely to cell apoptosis, which has become the domestic and foreign hot spot. NF-κB participates in transcriptional regulation of many genes related to apoptosis, such as IAPs, Bcl-2, Bcl-xL and c-myc, and it inhibits cell apoptosis(Dutta *et al.,*2006). With the thorough study on mechanism of NF-êB inhibiting cell apoptosis, people have found that

NF-êB can promote cell apoptosis when stimulated by different factors and being in specific cell types. The mechanism of NF-κB promoting apoptosis is concerned with increasing the expression of genes related to apoptosis, such as Fas and FasL(Bian *et al.,*2001; Campbell *et al.,*2004). In this study, we detected reporter gene dual luciferase activity using dual luciferase reporter system. The results showed that compared with the control group, Rh2 increased relative luciferase activity after treatment($P<0.05$), which showed Rh2 could induce the transcriptional activation of transcription factor NF-êB. On the base of this experiment, in order to further demonstrate that Rh2 could activate transcription factor NF-κB, we used immunofluorescence assay to test phosphorylation expression level and nuclear translocation of transcription factor NF-κB p65 to conduct positioning, characterization, quantitative analysis. Our result indicated that after A549 cells were treated by 20 μg/mL Rh2 for 0.5h-1h, compared with the control group, the fluorescence luminance value for marking NF-êB p65 phosphorylation expression level in the nucleus increased significantly ($P<0.05$), which indicated that Rh2 could up-regulate phosphorylation expression of transcription factor NF-κB p65 and promote it into nucleus. Thus, we conclude that NF-êB family and NF-κB-mediated signal transduction pathways are activated during the process of the human lung adenocarcinoma A549 cell apoptosis induced by Rh2.

TNF-α is cytokine related to apoptosis which is closely related to NF-κB-mediated cell signal transduction pathways(Cui *et al.,*2011). Zhou DB et al.(2007) reported that the concentration of TNF-á and NO in the bronchoalveolar lavage fluid (BALF) and the cultured supernatants of alveolar macrophages (AM) in 35 patients with NSCLC were measured by ELISA and enzyme method. The results showed that the activity of TNF-á and NO was lower in the BALF and the cultured supernatants of AM of the tumor-bearing lungs than that of the non- tumor-bearing lungs. However, the concentrations of TNF-α and NO in the cultured supernatants of AM cultivated with G-Rh2 were higher than those in the control ($p<0.05$). These results demonstrated that G-Rh2 can enhance the excretion of cytotoxin-effecting molecules of AM in patients with NSCLC. In order to illustrate farther that Rh2 can regulate NF-κB-mediated signal transduction pathway, and explore the possible mechanism of NF-κB regulating target gene expression and inducing A549 cell apoptosis, we detected TNF-α mRNA expression level by RT-PCR. Our result indicates Rh2 could promote TNF-α mRNA expression obviously.

Thus, the present study demonstrates that Rh2 contributes to induce the human lung adenocarcinoma A549 cell apoptosis, the molecular mechanism is related to activating of NF-êB signaling pathway and up- regulating the expression of its target gene TNF-á. However, cell apoptosis is a complex process, and different mechanisms are often intertwined. Previous researches have suggested that Ginsenoside-Rh2-induced tumor apoptosis signaling pathways are complicated and always involved in cross-talking among different pathways. It can cause tumor apoptosis by inducing cell cycle arrest or regulating ROS, Ca2+, PKC, or JNK-mediated cell signaling pathways and TRAIL-R1(DR4)-mediated death receptor pathways(Ham *et al.,*2006;Choi *et al.,*2007). Yet, as far as human lung adenocarcinoma A549 cell apoptosis induced by 20 (S)-Ginsenoside-Rh2 is concerned, signaling cross-talking between NF-κB, TNF-α and other pathways remain incompletely elucidated, thus our research results build up a solid foundation for the further research of the mechanism.

ACKNOWLEDGEMENTS

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