Ginsenoside Rh2 Inhibited Proliferation by Inducing ROS Mediated ER Stress Dependent Apoptosis in Lung Cancer Cells

Guanqun Ge, a Yan Yan, b and Hui Cai a,c

a Department of Breast Surgery, The First Affiliated Hospital of Xi’an Jiaotong University; Xi’an, Shaanxi 710061, China; b The Second Department of Thoracic Surgery, The First Affiliated Hospital of Xi’an Jiaotong University; Xi’an, Shaanxi 710061, China; and c Department of Vascular Surgery, The First Affiliated Hospital of Xi’an Jiaotong University; Xi’an, Shaanxi 710061, China.

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Ginsenoside Rh2 (G-Rh2), a component extracted from roots of ginseng, exhibited anti-cancer pharmacological activities by inhibiting proliferation and inducing apoptosis in lung cancer cells. However, the mechanisms of G-Rh2 suppressing lung cancer development remained elusive. This study tried to investigate the possible mechanism involved in anti-proliferative effect of G-Rh2 in lung cancer cells. As results, G-Rh2 inhibited the proliferation of H1299 cells in a dose-dependent manner by inducing cell apoptosis. Activating transcription factor 4 (ATF4), CCAAT/enhancer-binding protein homologous protein (CHOP), and caspase-4 were involved in G-Rh2-induced apoptosis of H1299 cells. It was also found that G-Rh2 could up-regulate expressions of ATF4, CHOP and caspase-4 in H1299 cells in a dose-dependent manner. In addition, NAC (N-acetylcysteine, a reactive oxygen species (ROS) scavenger) treatment dramatically decreased ROS generation in H1299 cells; both of NAC and 4-PBA (4-phenylbutyrate, a specific endoplasmic reticulum (ER) stress inhibitor) administration impaired apoptosis and expression levels of ATF4, CHOP and caspase-4 in G-Rh2 incubated H1299 cells. In vivo assays extended the significance of these results, showing that G-Rh2 inhibited lung cancer growth and the inhibition effects of G-Rh2 in tumor growth were significantly reduced by inhibition of ER stress. In conclusion, G-Rh2 inhibited proliferation of H1299 cells by inducing ROS mediated ER stress dependent cell apoptosis.

Key words ginsenoside Rh2; lung cancer; apoptosis; reactive oxygen species; endoplasmic reticulum stress

Followed by serial biological steps including hyperplasia, metaplasia, dysplasia and in situ carcinoma, similar to other cancers, lung cancer eventually develops its malignancy marked by strong proliferative capacity which is characterized by resistance to apoptosis.1) Nowadays, lung cancer is still among the top common cancers worldwide. According to the records, lung cancer comprises 12.7% of cancer and 18.2% of all cancer-related death.2) Though the multiple therapies including surgery, radiotherapy and chemotherapy are more and more widely applied to patients, the prognosis of lung cancer is still poor due to its malignant nature. Non-small cell lung cancer (NSCLC) takes approximately 85% of lung cancer cases with less than 20% 5-year survival rate.3) Seeking and identifying new drugs inducing apoptosis may provide us new insight into prevention and treatment of lung cancer.

As one of the vital organelles in eukaryotic cells, endoplasmic reticulum (ER) is responsible for directing correct protein folding, calcium homeostasis and lipid metabolism.4) However, under certain physiological and pathological conditions, the normal functions of ER were impaired by multiple extra- or intra-cellular stimuli, which is referred as ER stress.5) Reactive oxygen species (ROS) is considered as one of the stimuli inducing ER stress. This phenotype is called ROS-mediated ER stress.6) Many anticancer agents such as chemotherapeutic drugs are believed to induce apoptosis by generating excessive intracellular ROS which is related with unfolded protein response (UPR) in ER to induce ER stress.7) Numerous investigations supposed that ROS-mediated ER stress could further promote cell apoptosis by activating CCAAT/enhancer binding protein (CREB) homologous protein CHOP signaling pathway.8) As a pre-apoptotic factor, CHOP is suggested to activate caspase cascade by inducing cleavage of caspase-12 (rodents)9) or caspase-4 (human).10)

In recent decades, natural products gradually become new alternatives besides regular therapies. With a long history in traditional medicine in East Asia, ginseng (Panax quinquefolius) is now a very popular herb as medicine or food supplement in the world.11) Extracted from roots of ginseng, ginsenosides are the main effective components.12) It was suggested in the previous study that among the ginsenosides, ginsenoside Rh2 (G-Rh2, first isolated by Shizuo Odashima13) exhibited various biological and pharmacological activities including anti-inflammation, anti-diabetes and anti-cancer.14-17) A recent report supposed that G-Rh2 induced colorectal cancer cell apoptosis by inducing excessive intracellular ROS generation.18) Thus, ROS-mediated ER-stress induced apoptosis attracted our attention in investigating gensenoside Rh2’s mechanism of anti-proliferation.

In this present study, we showed G-Rh2 inhibited proliferation of lung cancer cells by inducing apoptosis. Furthermore, whether ROS-mediated ER-stress induced apoptosis is the mechanism of G-Rh2’s anti-proliferative effect was also assessed. Employments of N-acetyl cysteine (NAC) — the ROS scavenger and 4-phenylbutyric acid (4-PBA) — the ER stress specific inhibitor were used to strengthen our conclusions. Our data would be helpful in understanding G-Rh2’s anti-cancer mechanism and therefore support G-Rh2 as an ideal anti-cancer agent in lung cancer treatment.

MATERIALS AND METHODS

Cell Culture and Treatment Human lung cancer H1299
cells were purchased from China Center for Type Culture Collection (CCTCC, China) and maintained in RPMI 1640 medium (Hyclone, U.S.A.) supplemented with 10% fetal bovine serum (FBS, Gibco, U.S.A.) and 1% antibiotic solution (containing 150 µmol/L streptomycin and 100U/mL penicillin, Sigma-Aldrich, U.S.A.). Cells were cultured in an incubator (Thermo, U.S.A.) with 37°C, 5% CO₂, and saturated humidity. Same amount cells were incubated with G-Rh2, provide by LKT Laboratories (Cas No. 78214-33-2, St. Paul, MN, U.S.A.), at serial concentrations (dissolved in ethanol) for 24h. In some cases, same amount cells were pre-treated with 4-PBA (500 µmol/L, Sigma-Aldrich, U.S.A.) or NAC (1 mmol/L, Sigma-Aldrich) for 2h before G-Rh2 incubation.

**Cell Proliferation Assessment** Cell proliferation of H1299 cells was assessed by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, U.S.A.) assay. 1 × 10⁴ cells/mL were planted in a 96-well plate (Corning, U.S.A.) and maintained for 24h. Then cells were wished by phosphate buffered saline (PBS) twice and incubated with MTT (Sigma-Aldrich, 5 mg/mL) for 4h. After dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to each well, the values of absorbance at 450 nm (A₄₅₀) were read by a plate reader (Bio-rad, U.S.A.). The inhibition of proliferation was presented as inhibitory rate (the ratio of nonviable cell in experimental wells compared to control wells).

**Intracellular ROS Determination** The intracellular ROS generation was determined by flow cytometry after 2,7-dichlorofluoresceindiacetate (DCFH-DA, Beyotime, China) staining. Treated cells were incubated with DCFH-DA (Beyotime, China) at final concentration of 10 µmol/L for 30 min at 37°C in an incubator. Then the fluorescence of 2,7-dichlorofluorescein (DCF, Beyotime, China) was captured by a flow cytometer (FACS Calibur, BD, U.S.A.) at 530 nm after the cells were excited by laser at wave length of 488 nm.

**Cell Apoptosis Detection** The apoptosis cells were distinguished from normal and necrotic one by using annexin V-propidium iodide (PI) apoptosis detection kit (Santa Cruz, U.S.A.) per manufacturers’ instructions. The cells were washed by PBS then suspended in annexin V-fluorescein isothiocyanate (FITC) binding buffer and incubated in a dark compartment for 10 min. Then the sample was incubated with PI before cytometric analysis by using a flow cytometer (FACS Calibur, BD, U.S.A.). Cell Quest pro software (BD, U.S.A.) was used to analyze the data.

**RNA Transfection** Six-well plates were seeded with 5 × 10⁴ cell/well in 2 mL media 24h before transfection; cells were 80–90% confluent. Cells were transfected with small interfering RNA (siRNA) (100 pmol/well) using Lipofectamine 2000 Reagent (Life Technologies, U.S.A.) according to manufacturer’s instruction. After 48h of transfection, cells were used for qRT-PCR or apopsis assay. All siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, U.S.A.).

**Western Blotting** The protein expression levels of activating transcription factor 4 (ATF4), CHOP and caspase-4 were evaluated by Western blotting. H1299 cells were collected after being washed by cold PBS twice then resuspended in RIPA lysis buffer system (Santa Cruz, U.S.A.) supplemented with 1× protease inhibitor cocktail (Invitrogen, U.S.A.). Total protein was collected and protein concentration was detected by BCA kit (Invitrogen). Then after separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein sample was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, U.S.A.) which were then incubated with antibodies against ATF4 (1 : 1000, Abcam, U.S.A.), CHOP (1 : 1000, Abcam), caspase-4 (1 : 1000, Abcam).
CST, U.S.A.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Santa Cruz, U.S.A.) at 4°C for 12 h in TBST containing 5% defatted milk. After being washed, the membranes were then incubated with corresponding second antibodies conjugated with horseradish peroxidase (Santa Cruz) for 1 h at room temperature. The bound antibodies were visualized on a LAS 4000 imaging system (FUJIFILM, Japan) by using Western blotting Luminal Reagent (Santa Cruz).

**Tumor Xenograft Experiments** All experiments involving mice were approved by Institutional Research Committee of Xi’an Jiaotong University. All mice received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. 2.5 million cancer cells were mixed in a 1:1 (v/v) ratio with Growth Factor Reduced Matrigel (BD, U.S.A.), and themixture was injected subcutaneously into the right flanks of 6- to 7-week-old BALB/c nu/nu nude mice. After implantation for 24h, mice bearing H1299 cells were randomly assigned to 6 groups as follows: 1) control, 2) 20 mg/kg/d NAC alone, 3) 20 mg/kg/d 4-PBA alone, 4) 20 mg/kg/d G-Rh2, 5) 20 mg/kg/d G-Rh2 combined with pre-treatment of NAC, 6) 20 mg/kg/d G-Rh2 combined with pre-treatment of 4-PBA. Each group consisted of 10 mice. G-Rh2, NAC, and 4-PBA suspended in saline, were intraperitoneally injected once a day. Controls received the vehicle alone (20 mL/kg). Tumor volume (in mm³) was determined by caliper measurements performed every 2d and calculated by using the modified ellipse formula: (volume=length×width²/2). Mice were sacrificed by cervical dislocation on day 40. Implanted tumors were separated and weighed. Expression of ATF4, CHOP and caspase-4 in tumors were evaluated by Western blotting.

**Statistical Considerations** The data acquired in this study was expressed as (mean±standard deviation (S.D.)) and processed by SPSS (ver. 16.0) statistical software. One way ANOVA and Student’s t-tests were employed to analyze the significance of differences in groups and between groups. p<0.05 was considered to be significant statistically.

**RESULTS**

G-Rh2 Dose-Dependently Inhibited Proliferation of H1299 Cells by Inducing Apoptosis We tested whether G-Rh2 affected the proliferation of H1299 cells. As demonstrated in Fig. 1A, results from the MTT assay showed that the administration of G-Rh2 at serial concentrations (0, 10, 20, 30, 40, 50 µmol/L) inhibited cell viability in a dose-dependent manner. G-Rh2 began to show significant inhibitory effect at 30 µmol/L. The notion was generally accepted that the reduction of proliferation was due to enhanced apoptosis induction. As shown in Fig. 1B, results from flow cytometry

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Fig. 2. Effects of G-Rh2 on Expressions of ATF4, CHOP and Caspase-4

(A) Reduced ATF4, CHOP and caspase-4 expression by specific siRNA in H1299 cells detected by Western blots. (B) The effect of down-regulation of ATF4, CHOP and caspase-4 on cell apoptosis induced by G-Rh2 (50 µmol/L for 24h). in H1299 cells. The results are from three independent experiments. a, b, c, and d values are significantly different from control (p<0.05); a, b, and c values are significantly from G-Rh2 (p<0.05). (C) Immunoblots of ATF4, CHOP, caspase-4 and GAPDH were shown correspondingly in H1299 cells incubated with G-Rh2 at serial concentrations (0, 10, 20, 30, 40 and 50 µmol/L). Columns indicated relative expressions (normalized to GAPDH) of ATF4, CHOP and caspase-4 in H1299 cells incubated with G-Rh2 at serial concentrations (0, 10, 20, 30, 40 and 50 µmol/L). Values are represented in a (means±S.D.) manner. *Values are significantly different when compared with lower concentration (p<0.05). Independent experiments were repeated 3 times.
demonstrated that the apoptosis rate increased significantly after incubated with G-Rh2 (at concentrations of 0, 10, 20, 30, 40, 50 µmol/L) in a dose-dependent manner.

**G-Rh2 Induced Apoptosis through ATF4/CHOP/Caspase-4 Signaling Pathway in H1299 Cells**

ATF4 is the molecular marker of intensified ER stress. CHOP is a pro-apoptotic factor which conducting apoptosis signaling in ER stress to caspase-4 to initiate apoptosis by activating caspase cascade. Specific siRNAs were used to dissect the signaling pathway leading to the G-Rh2 effects on apoptosis. Selective blockage of ATF4, CHOP and caspase-4 was achieved utilizing specific siRNAs as assessed by Western blots (Fig. 2A). Down-regulation of ATF4, CHOP and caspase-4 reversed G-Rh2-induced apoptosis in H1299 cells (Fig. 2B). Furthermore, we also tested effects of G-Rh2 on expression of ATF4, CHOP and caspase-4 in H1299 cells. As shown in Fig. 2C, G-Rh2 increased the expressions of all these three proteins in a dose-dependent manner.

**Effects of NAC and 4-PBA Pre-treatment on Apoptosis Induced by G-Rh2 in Cultured H1299 Cells**

NAC is generally used as a ROS scavenger which could eliminate ROS by its anti-oxidant property. 4-PBA is a molecular chaperon assisting correct protein folding process in ER lumen which is therefore usually used as a specific ER stress inhibitor. In order to testify our presupposition, apoptosis-inducing effects of G-Rh2 were observed in NAC and 4-PBA pre-treated H1299 cells, respectively.

NAC Reversed G-Rh2 Induced Intra cellular ROS Generation, ATF4/CHOP/Caspase-4 Expressions and Apoptosis in H1299 Cells

As demonstrated in Fig. 3A, NAC pre-treatment significantly decreased intracellular ROS levels in G-Rh2 treated cells. The apoptosis rate was also reduced by NAC pre-treatment in G-Rh2 incubated H1299 cells (Fig. 3B). Furthermore, the NAC pre-treatment significantly decreased ATF4, CHOP and caspase-4 expression levels at translational level induced by G-Rh2 (Fig. 3C).

4-PBA Reversed ATF4, CHOP and Caspase-4 Expressions and Apoptosis Induced by G-Rh2

As shown in Fig. 4A, 4-PBA pre-treatment dramatically decreased apoptosis rate in G-Rh2 treated H1299 cells. Figure 4B showed that 4-PBA pre-treatment also down-regulated expressions of ATF4, CHOP and caspase-4 in H1299 cells. However, 4-PBA did not reverse the effect of G-Rh2 in inducing intracellular ROS generation, indicating ER stress as downstream of ROS (Fig. 4C).

**G-Rh2 Inhibited Lung Cancer Cell Proliferation by Inducing ROS Mediated ER Stress Dependent Apoptosis in Vivo**

To investigate the in vivo functions of G-Rh2 in tumors on growth, we generated subcutaneous tumors in BALB/c nu/nu nude mice using H1299 cells. Consistent with in vitro results, tumors treated with G-Rh2 grew slower than control tumors (Fig. 5A). However, the inhibition effects of G-Rh2 in tumor growth were significantly reduced by pre-treating with NAC and 4-PBA (Fig. 5A). Forty days after tumor initiation, tumors were removed and macroscopically measured. As shown in Fig. 5B, G-Rh2 could significantly reduce tumor volume and this effect was inhibited by pre-treatment with NAC and 4-PBA. Moreover, G-Rh2 could induced over-expression of ATF4, CHOP, and caspase-4 through ER stress in vivo (Fig. 5C).
DISCUSSION

Patients diagnosed as NSCLC which account the majority of lung cancer are usually described with poor prognosis and life quality. In recent decades, the morbidity and mortality of lung cancer are climbing quickly worldwide accompanied by the process of industrialization. Except for the patients diagnosed at the very early stage, potentially curative therapy, surgery, are not applicable in patients with progressed lung cancer. Chemotherapy and radiotherapy are then used to...
control and inhibit the malignant proliferation of lung cancer cells but the prognosis is still poor.\(^{23}\) New alternative therapeutic agents targeting proliferation of cancer cells are attracting attention in recent years. In this study, we reported that G-Rh2 inhibited proliferation of H1299 human lung cancer cells by inducing apoptosis. In depth, we also investigated the possible mechanisms of G-Rh2’s anti-proliferation effects. Finally, we concluded that G-Rh2 induced apoptosis of H1299 cells by stimulating ROS-mediated ER stress induced apoptosis.

G-Rh2 is one of the main bioactive components extracted from ginseng roots exhibiting various biological activities including anti-inflammation, anti-diabetes and anti-cancer effects. Previous studies reported that G-Rh2 could inhibit proliferation, invasion and metastasis of multiple human cancers.\(^{24,25}\) Inducing apoptosis in cancer cells is an important mechanism of many anti-cancer agents in impairing malignant proliferation. In this study, we found expressions of ATF4, the molecular marker of ER stress, CHOP and caspase-4 were significantly up-regulated in G-Rh2 incubated H1299 cells in a concentration-dependent manner. This result indicated that G-Rh2 could induce apoptosis of H1299 cells by activating ER stress dependent pathway.

In this study, we found that ER stress induced apoptosis also was an alternative pathway that G-Rh2 could induce cell death in lung cancer.

ER is an organelle conducting and providing environment for protein folding in eukaryotic cells. Because its sensitivity to stimuli which would induce a condition called ER stress.\(^{29}\) The UPR signals are activated to achieve remission of the stress and regain homeostasis of ER. However, severe and sustained ER stress would further lead apoptosis by inducing activation of pro-apoptotic factor CHOP which would activate caspase-4 and leading to caspase cascade activation.\(^{30}\) This process is called ER-stress dependent apoptosis.

As an important biological modulator, ROS not only play a role in maintaining normal physiological functions but also participate in cell damage and cell death because of their highly intensive activities.\(^{31}\) As one of the adverse stimuli, ROS may cause dysfunction of ER and induce ER stress,
which is called ROS-mediated ER stress. In the present study, the H1299 cells were pre-treated by ROS scavenger NAC before incubation with G-Rh2. Alleviation of apoptosis was found, accompanied by attenuation of ROS generation and down-regulation of ATF4, CHOP and caspase-4 expressions. This result indicated that ROS are indispensable for G-Rh2 induced ER stress dependent apoptosis in H1299 cells.

CONCLUSION

In summary, we demonstrated that G-Rh2 could inhibit proliferation of H1299 cells by inducing apoptosis, and the apoptosis was induced by ROS mediated ER stress dependent pathway. These findings might be helpful in expanding understanding of the mechanisms of anti-cancer pharmacological effects of G-Rh2. More importantly, these understanding would provide theoretical basis for developing new and more effective G-Rh2 based anti-cancer drugs.

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Conflict of Interest The authors declare no conflict of interest.

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