Ginsenoside Rh2 inhibits HeLa cell energy metabolism and induces apoptosis by upregulating voltage-dependent anion channel 1

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Abstract. 20(S)-Ginsenoside Rh2 [20(S)-GRh2], one of the main active components of Panax ginseng, induces apoptosis in a wide range of cancer cell types. The present study found that 20(S)-GRh2 reduces mitochondrial membrane potential, decreases adenosine triphosphate generation and induces reactive oxygen species in HeLa cervical cancer cells. In addition, 20(S)-GRh2 activated mitochondrion-dependent apoptosis and inhibited both mitochondrial oxidative phosphorylation and glycolysis in HeLa cells. It was found that voltage-dependent anion channel 1 (VDAC1) expression was significantly upregulated by 20(S)-GRh2 treatment, while hexokinase 2 expression was downregulated and segregated from the mitochondria. Furthermore, 20(S)-GRh2 promoted Bax transport from the cytoplasm to the mitochondria, and knockdown of VDAC1 inhibited Bax transport and apoptosis. These results suggest that VDAC1 is a novel target of 20(S)-GRh2. The present study provides a better understanding of the mechanistic link between cervical cancer metabolism and growth control, and these results may facilitate the development of new treatments for cervical cancer.

Introduction

Cervical cancer is the fourth most common gynecological malignant tumor that affects females, and results in ~57,000 new cases annually with 311,000 deaths worldwide (1,2).

In China, the incidence of cervical cancer has increased rapidly over the past 20 years (3). Surgery, radiotherapy and chemotherapy are currently the main treatment methods for cervical cancer (4). Although advances in these therapies have prolonged patient survival, the limitations of effective regimens are the resistance of tumor cells and their severe side effects (5). Thus, there is an urgent need for new anticancer drugs with fewer side effects that may improve survival rates.

Voltage-dependent anion channel (VDAC) family proteins in the mitochondrial outer membrane play an important role in cell survival and death (6). VDACs interact with members of the Bcl-2 family to participate in the release of cytochrome c and the subsequent activation of caspase-9 (7,8). Furthermore, VDACs are involved in caspase-independent apoptosis (9). Among the three isomers (VDAC1, VDAC2 and VDAC3), VDAC1 shows the highest expression in mammalian mitochondria and plays an important role in apoptosis (10,11). VDAC1 upregulation promotes apoptosis in multiple cell lines (12,13). VDAC1 is also a target of various pro-apoptotic compounds such as curcumin, arsenic trioxide and cannabinoids (14-16).

A previous study showed that a VDAC1-based peptide induces apoptosis by releasing bound hexokinase 2 (HK2) from the mitochondria (17). HK2 is a crucial enzyme in the glycolytic pathway and antagonizes apoptosis through its direct interaction with VDAC1 (18). HK2 is overexpressed in a variety of tumor cell types, and HK2 is transported to the mitochondria and interacts with VDAC1, which promotes the development of anaerobic metabolism to compensate for the higher energy requirements in tumor cells (19). Most tumor cells still show active glucose uptake and glycolysis under aerobic conditions, a phenomenon known as the Warburg effect, and metabolic dysregulation affects tumor growth and cell death (20,21). Several compounds, such as 3-bromopyruvate and methyl jasmonates, interact with HK2, causing HK2 to separate from mitochondria and trigger apoptosis (22,23).

20(S)-Ginsenoside Rh2 [20(S)-GRh2], a protopanaxadiol-type ginsenoside extracted from Panax ginseng, is an active component with an anti-tumor effect that inhibits tumor cell growth and induces tumor cell apoptosis (24,25). Previous studies showed that 20(S)-GRh2 has safety advantages (such as reverse multidrug resistance, balanced immunity and
enhanced chemotherapy) (24) and shows no side effects, indicating 20(S)-GRh2 may be a promising new antitumor drug (26). Several reports demonstrated that 20(S)-GRh2 stimulates apoptosis and induces mitochondrial dysfunction by activating caspases, upregulating various pro-apoptotic proteins such as Bax and downregulating Bcl-XL (27-29). Therefore, 20(S)-GRh2 may stimulate HeLa cell apoptosis by affecting the mitochondria and regulating energy metabolism.

The present study tested this hypothesis and the results showed that 20(S)-GRh2 exposure activated mitochondrion-dependent apoptosis and suppressed mitochondrial oxidative phosphorylation and glycolysis. To understand its underlying mechanism, it was found that VDAC1 was significantly upregulated in response to 20(S)-GRh2 treatment, while HK2 was downregulated and separated from mitochondria. Bax transport from the cytosol to the mitochondrion was promoted by 20(S)-GRh2 and knockdown of VDAC1 reduced 20(S)-GRh2-induced Bax translocation and apoptosis. The results provided a more comprehensive understanding of the mechanistic association between cancer mitochondrial metabolism and tumor growth control by 20(S)-GRh2 and may facilitate the development of new therapeutic methods for cervical cancer.

Materials and methods

Materials and antibodies. 20(S)-GRh2 (98% pure) was purchased from Shanghai Yanye Biotechnology Co., Ltd. The following primary antibodies were used in this study: Anti-HK2 rabbit polyclonal antibody (1:1,000; cat. no. 22029-1-AP; ProteinTech Group, Inc.), anti-VDAC1 rabbit monoclonal antibody (1:1,000; cat. no. AF1027; Beyotime Institute of Biotechnology), anti-prohibitin rabbit monoclonal antibody (1:1,000; cat. no. AF1126; Beyotime Institute of Biotechnology), anti-cytochrome c rabbit monoclonal antibody (1:1,000; cat. no. AF2047; Beyotime Institute of Biotechnology), anti-Bax rabbit monoclonal antibody (1:1,000; cat. no. D2E11; Cell Signaling Technology, Inc.), anti-Bcl-2 rabbit polyclonal antibody (1:1,000; cat. no. abs131701, Absin) and anti-β-actin mouse monoclonal antibody (1:1,000; cat. no. BS6007M; Bioworld Technology, Inc.).

Cell culture and transfection. HeLa cells were obtained from the Cell Bank of the Chinese Academy of Sciences and cultured in DMEM (HyClone; Cytiva) with 10% FBS (HyClone; Cytiva), 100 µg/ml penicillin and 100 µg/ml streptomycin (HyClone; Cytiva) at 37˚C in a humidified atmosphere with 5% CO₂. Cells were transfected with a final concentration of 100 nM small interfering RNA (siRNA) targeting VDAC1 using riboFECT CP reagent (Guangzhou Ribobio co., Ltd.) according to the manufacturer’s instructions. Following incubation for 24 h, HeLa cells were treated with 20(S)-GRh2 (35 and 45 µM) for 24 h at 37˚C before subsequent experimentation. The target sequence in negative controls for RNA interference is 5’-AAT TCTCCGAACGTGTCACGT-3’; and the target sequences in VDAC1 for RNA interference were 5’-GGAGACCGCTGTCAATCTT-3’ (siVIC1-1) and 5’-GCTGCGACATGGATTTCGA-3’ (siVIC1-2).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from HeLa cells treated with 20(S)-GRh2 using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA purity was measured using a BioSpec-nano spectrophotometer (Shimadzu Corporation), and total RNA was reverse transcribed using a PrimerScript RT Reagent Kit with gDNA Eraser (Takara Bio, Inc.) according to the manufacturer’s instructions. RT-qPCR was performed using a SYBR-Green assay (Takara Bio, Inc.) on a CFX Connect Real-Time system (Bio-Rad Laboratories, Inc.). Fold-changes in the expression of each gene were calculated using the 2^-ΔΔcq method (30). The following thermocycling conditions were used for the qPCR:

Initial denaturation at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. GAPDH mRNA was used as an internal reference. The following primer pairs were used for the qPCR: GAPDH forward, 5’-CTGGGCTCAGACTGACCA-3’ and reverse, 5’-AAGTGTGGTGTAGGGCAATG-3’ (Harbin Xinhai Genetic Testing Co., Ltd.); VDAC1 forward, 5’-CTGACCTTCGATTCACTCTTC-3’ and reverse, 5’-CTCCCGTTGTAGCCCTGC-3’ (Harbin Xinhai Genetic Testing Co., Ltd.) and HK2 forward, 5’-AGCCACACCAATCCATCATTAC-3’ and reverse, 5’-CCATTGCGTTACTTTCAC-3’ as well as forward, 5’-AGCCACACCTACACTAC-3’ and reverse, 5’-CCCATTGTCGGTTACTTTC-3’ (Comate Bioscience Co., Ltd.).

Cell viability assessment. The Cell Counting Kit-8 (CCK-8) assay (Wuhan Boster Biological Technology Co., Ltd.) was used to assess cell viability according to the manufacturer’s instructions. HeLa cells were treated with 20(S)-GRh2 at 37°C for 24 h at various concentrations (35, 45, 55 and 65 µM) to determine the IC₅₀ values. In addition, cells were treated with IC₅₀ concentrations at 37°C at various timepoints (6, 12, 24, 36 and 48 h). Following treatment, 20 µl CCK-8 solution was added to each well, and the plate was incubated at 37°C for 1 h. Absorption at a wavelength of 450 nm was recorded using an Infinite M200 pro reader (Tecan Group, Ltd.) and the cell viability was calculated.

Cell apoptosis analysis. Cells were treated with various concentrations (35 and 45 µM) of 20(S)-GRh2 at 37°C for 24 h. Cells were then suspended in 1X Binding Buffer and incubated with 5 µl Annexin V-FITC and 5 µl PI (BD Biosciences) at room temperature in the dark for 15 min. The cells were analyzed with a Flow Sight flow cytomter (Amnis Corporation) and data were analyzed using IDEAS software v6.1 (Amnis Corporation). Apoptotic cells were also analyzed by Hoechst 33342 staining (Beyotime). Briefly, following 20(S)-GRh2 (35 and 45 µM) treatment, cells were incubated with Hoechst 33342 at 37°C in the dark for 30 min. Cells treated with 45 µM DMSO were used as the control group. Images were captured under an EVOS FL Auto fluorescence microscope (Thermo Fisher Scientific, Inc.) at x295 magnification.

Determination of the mitochondrial membrane potential (MMP). MMP alterations were measured using a JC-1 MMP Assay Kit (Beijing Solarbio Science & Technology Co., Ltd.). HeLa cells were treated with 20(S)-GRh2 (35 and 45 µM) at 37°C for 24 h and then washed with cold PBS. A total of 10 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used to induce a decrease in mitochondrial membrane potential for
20 min as a positive control. JC-1 working solution was added to the cell culture medium, and cells were incubated at 37°C in the dark for 20 min. Cells were imaged using an EVOS FL Auto fluorescence microscope (Thermo Fisher Scientific, Inc.) at x295 magnification.

MMP was also measured using the cationic dye rhodamine 123 (Beyotime Institute of Biotechnology). Briefly, HeLa cells were pre-treated with 20(S)-GRh2 (35 and 45 µM) at 37°C for 24 h and then incubated with 2 µM rhodamine 123 at 37°C in the dark for 30 min. Fluorescence intensity was detected using the Flow Sight flow cytometer (Amnis Corporation) and data were analyzed using IDEAS software v6.1 (Amnis Corporation).

Western blotting. Cells treated with 20(S)-GRh2 (35 and 45 µM) at 37°C for 24 h were lysed in RIPA buffer (Beyotime Institute of Biotechnology) pre-cooled at 4°C. Mitochondrial and cytoplasmic proteins were extracted using a cell mitochondrial isolation kit (Beyotime Institute of Biotechnology). Protein quantification was performed using the BCA method (Beyotime Institute of Biotechnology). Equal amounts of protein (30 µg) were subjected to 12% SDS-PAGE and then transferred onto a nitrocellulose membrane (Pall Corporation). The membranes were blocked with 5% skim milk powder and PBS for 1 h at room temperature, then incubated with primary antibodies overnight at 4°C, followed by incubation with goat polyclonal secondary antibody to mouse IgG-H&L (HRP) (1:1,100; cat. no. 115-005-00; Jackson ImmunoResearch Laboratories, Inc.) and goat anti-rabbit IgG H&L (HRP) (1:1,100; cat. no. 115-005-003; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at 37°C. Development was performed with an ECL kit (Beyotime Institute of Biotechnology). Imaging and densitometric analysis were performed using an iBright FL1000 imaging system (Invitrogen; Thermo Fisher Scientific, Inc.). β-actin and prohibitin were used as loading controls.

Measurement of cellular ATP levels. Total ATP levels were determined using a Chemiluminescence ATP assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, the culture solution was removed, followed by addition of 200 µl lysis to each well of a six-well plate and centrifuged at 12,000 x g to collect the cell supernatant. In addition, 100 µl ATP detection working solution was added to the 96-well assay plates and incubated at room temperature for 5 min. Subsequently, 20 µl sample was added to the wells and analyzed on the Infinite M200 pro reader (Tecan Group, Ltd.). 

Mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis assays. A total of 5x10^4 HeLa cells were seeded into the B-G pores of an XF 8 Cell Culture Microplate (Agilent Technologies, Inc.) in 80 µl medium. Equal volumes of media were then added into the A and H pores, followed by overnight incubation at 37°C with 5% CO2. Only the calibration solution was left on both sides of the culture chamber. HeLa cells were treated with 20(S)-GRh2 (35 and 45 µM) for 24 h. The probe plate of the Seahorse XFp sensor was incubated overnight in the calibration solution the day before the experiment. Cells were washed with 180 µl DMEM without bicarbonate and then incubated at 37°C for 1 h before measurement.

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using an XFp Extracellular Flux Analyzer (Agilent Technologies, Inc.) according to manufacturer's instructions. The OCR was continuously recorded for 12 cycles, with each cycle consisting of 3 min of mixing and 3 min of measurement. Basal respiration was measured during cycles 1-3. Further measurement of OCR was run as follows: As 4-6 cycles of oligomycin injection (oligo, ATP synthase inhibitor, 5 µM), 7-9 cycles of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, proton, 5 µM) injection and 10-12 cycles of rotenone/antimycin A (AA/Rot, complex I and III inhibitor, 2.5 µM) injection [all, XFp Cell Mito Stress Test kit (Agilent Technologies, Inc.)]. Further measurement of ECAR was run as follows: 4-6 cycles of glucose (substrate for hexokinase, 10 mM) injection, 7-9 cycles of oligomycin (oligo, ATP synthase inhibitor, 5 µM) injection and 10-12 cycles of 2-Deoxy-D-glucose (2-DG, competitive inhibitor of hexokinase, 50 mM) injection. The use of these inhibitors permits the determination of key aspects of mitochondrial function (31).

Caspase activity assays. Assays for caspase-3 (cat. no. C1151), -8 (cat. no. C1151) and -9 (cat. no. C1157) activities were performed using kits according to the manufacturer's instructions (Beyotime Institute of Biotechnology). Briefly, cells were washed with PBS, collected by centrifugation (600 x g, 5 min) at 4°C and resuspended in ice cold lysis buffer. A total of 100 µl lysis was used per 2x10^6 cells and incubated on ice for 20 min. The protein concentration in the supernatant was measured with a Bradford assay kit (Tiangen Biotech Co., Ltd.). Subsequently, 50 µl cell lysate supernatant was mixed with 10 µl Ac-DEVD-pNA (2 mM) for caspase-3, Ac-IETD-pNA (2 mM) for caspase-8 and Ac-LEHD-pNA (2 mM) for caspase-9 in 40 µl assay buffer for 4 h at 37°C, and then analyzed with an Infinite M200 pro reader (Tecan Group, Ltd.).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software 6 (GraphPad Software, Inc.). In quantitative analyses shown in histograms, values were obtained from three independent experiments and expressed as the mean ± SD. One-way ANOVA followed by Dunnett's post-hoc test was used to compare between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

20(S)-GRh2 inhibits cell viability and induces apoptosis in HeLa cells. The CCK-8 method was used to examine the effect of 20(S)-GRh2 (Fig. 1A) on HeLa cell viability. Cells
were treated with various doses of 20(S)-GRh2 for different timepoints. The results showed that 20(S)-GRh2 reduced the viability of HeLa cells in dose- and time-dependent manners (Fig. 1B and C). The IC₅₀ of 20(S)-GRh2 was calculated to be ~45 µM, and 35 and 45 µM for further experiments.

It was next examined whether 20(S)-GRh2 inhibited cell viability via inducing apoptosis. Hoechst staining showed an increase in the amount of condensed and fragmented nuclei in cells treated with 45 µM 20(S)-GRh2 compared with the control group (Fig. 1D and E), indicating that 20(S)-GRh2 induced apoptosis in HeLa cells. Annexin V and PI double staining was used to detect the apoptotic rate of HeLa cells treated with 20(S)-GRh2. The percentages of apoptotic cells upon treatment with 0, 35 and 45 µM 20(S)-GRh2 were 3.84, 12.38 and 52.04%, respectively (Fig. 1F and G). Taken together, these findings suggested that 20(S)-GRh2 reduces cell viability by inducing apoptosis in HeLa cells.

20(S)-GRh2 reduces MMP, decreases ATP generation and induces ROS in HeLa cells. Mitochondria play a central role in the growth and apoptosis of cells (32). Therefore, it was next assessed whether 20(S)-GRh2-induced apoptosis was associated with mitochondrial dysfunction. The effects of 20(S)-GRh2 on the MMP, an indicator of mitochondrial functions, were assessed using JC-1 and rhodamine-123 staining. Fluorescence microscopy showed that cells exposed to 20(S)-GRh2 showed increased conversion of JC-1 aggregates (red) to JC-1 monomers (green). Following treatment with 10 µM CCCP as the positive control, the cells showed obvious green fluorescence, indicating that 10 µM CCCP almost completely depleted the MMP of HeLa cells (Fig. 2A). Flow cytometry showed that 45 µM 20(S)-GRh2 treatment significantly reduced the fluorescence intensity of rhodamine-123 (Fig. 2B). MMP and ATP generation are indicators of mitochondrial function (33), and thus the effects of 20(S)-GRh2 on the cellular ATP levels were assessed. Compared with the control group, 20(S)-GRh2 exposure reduced cellular ATP levels in HeLa cells by 14% (35 µM) and 49% (45 µM) (Fig. 2C). The decrease in MMP and ATP levels showed that 20(S)-GRh2 induced mitochondrial dysfunction in HeLa cells.

It was hypothesized that 20(S)-GRh2-induced mitochondrial dysfunction may cause increased levels of ROS. To test this hypothesis, ROS levels in 20(S)-GRh2-treated HeLa cells were measured. Low concentrations of 20(S)-GRh2 (35 µM) had no significant effect on cellular ROS, whereas...
a high concentration of 20(S)-GRh2 (45 µM) significantly increased cellular ROS by 2.69-fold compared with control cells (Fig. 2d).

**20(S)-GRh2 inhibits mitochondrial OXPHOS and glycolysis.** Since the mitochondrial electron transfer chain is the main target site of ROS (34), the present study hypothesized that 20(S)-GRh2 might affect mitochondrial ATP generation, which is involved in 20(S)-GRh2-induced cell death. The XFp extracellular flux analyzer was then used to assess the role of 20(S)-GRh2 on mitochondrial OXPHOS and glycolysis, the two major energy metabolism pathways, in real-time. OXPHOS was measured by quantifying the OCR of the medium and glycolysis was assessed by quantifying the ECAR of the medium. Treatment with 20(S)-GRh2 (35 and 45 µM) caused a decrease in the OCR and altered cellular responses to typical mitochondrial complexes inhibitors (including oligomycin, an ATP synthase inhibitor; FCCP, a mitochondrial uncoupler and AA/Rot, inhibitors of complex I and III), suggesting that the mitochondrial OXPHOS capacity was inhibited (Fig. 3A).

HeLa cells treated with 20(S)-GRh2, the OCR associated with non-mitochondrial respiration was also reduced (Fig. 3A). This result indicated that 20(S)-GRh2 may also inhibit the oxygen consumption of the non-mitochondrial respiratory pathway, but the exact molecular mechanism was unclear.

Numerous cancer cells break down glucose by aerobic glycolysis to obtain the energy needed for growth in a process known as the Warburg effect (35). It was next examined whether 20(S)-GRh2 also induced this effect on glycolysis in HeLa cells. The results showed that both basal and stimulated ECAR reduced after 45 µM 20(S)-GRh2 treatment (Fig. 3C). 20(S)-GRh2 (45 µM) significantly reduced the ECAR linked to glycolytic capacity and the glycolytic reserve in HeLa cells (Fig. 3D). At 35 µM, 20(S)-GRh2 had no effect on ECAR (Fig. 3C) but inhibited OCR (Fig. 3A) and decreased cellular ATP levels (Figs. 2C and 3B), suggesting that 20(S)-GRh2 decreased ATP production primarily by inhibition of OXPHOS.

**Mitochondrial pathway is involved in 20(S)-GRh2-induced HeLa cell apoptosis.** To further assess the apoptotic process induced by 20(S)-GRh2, the activation of caspase-3, a critical marker of apoptosis, was investigated. As shown in Fig. 4A,
20(S)-GRh2 treatment significantly increased caspase-3 activity in a dose-dependent manner. Next, the upstream regulators of caspase-3 involved in 20(S)-GRh2-induced apoptosis were determined. Mitochondrion-associated caspase-9 activity was significantly increased by 45 µM 20(S)-GRh2 but did not affect death receptor-associated caspase-8 activity in HeLa cells (Fig. 4B and C). Since caspase-9 is activated by Bcl-2 family proteins (36), it was investigated whether 20(S)-GRh2 affected the expression of Bcl-2 and Bax proteins. 45 µM 20(S)-GRh2 induced Bax upregulation and Bcl-2 downregulation (Fig. 4d). Bcl-2 and Bax induce the release of cytochrome c from the mitochondria into the cytoplasm (37). Western blot analysis showed that 20(S)-GRh2 significantly promoted the release of cytochrome c into the cytoplasm, which coincided with caspase-9 activation (Fig. 4E). Once cytochrome c is released into the cytoplasm, it activates caspase-9, triggering a cascade causing mitochondrial-mediated apoptosis (38); hence, the results indicated that 20(S)-GRh2 induces apoptosis via the mitochondrial pathway (Fig. 4E).

Upregulation of VDAC1 plays a role in 20(S)-GRh2-induced apoptosis. VDAC1 plays an important role in regulating mitochondrial energy metabolism and mitochondrial-mediated apoptosis (39). Therefore, the role of VDAC1 in 20(S)-GRh2-induced apoptosis was assessed. VDAC1 expression in HeLa cells was significantly increased by 45 µM 20(S)-GRh2 treatment (Fig. 5A and B), indicating that induction of VDAC1 may be involved in 20(S)-GRh2-mediated induction of apoptosis in HeLa cells. To determine the role of VDAC1 in HeLa cell apoptosis induced by 20(S)-GRh2, a synthetic siRNA targeting VDAC1 to knockdown VDAC1 in HeLa cells was used (Fig. 5C and D). Annexin V staining showed that 20(S)-GRh2-induced apoptosis was suppressed in VDAC1-silenced cells (Fig. 5E and F). These results showed that 20(S)-GRh2 induced apoptosis in HeLa cells by regulating VDAC1.

20(S)-GRh2-induced reduction of HK2 results in VDAC1 upregulation. To further elucidate the molecular mechanism underlying 20(S)-GRh2-induced VDAC1 upregulation on HeLa cell apoptosis, known VDAC1-binding proteins were investigated. HK2 interacts with VDAC1 and plays major roles in tumor cell metabolism, including mitochondrial ATP synthesis and glucose metabolism (40). HK2 also inhibits tumor cell apoptosis by inhibiting changes in mitochondrial membrane permeability (41). Therefore, HK2 expression was measured. It was found that the expression of mitochondrial HK2 and cytosolic HK2 in HeLa cells treated with 45 µM 20(S)-GRh2 was decreased which was consistent with the pattern of total HK2 (Fig. 6A-C). By contrast, the expression levels of VDAC1 protein was increased in response to 45 µM 20(S)-GRh2 (Fig. 6D), indicating that 20(S)-GRh2 induces changes in the expression levels of HK2 and VDAC1 that are closely related to its induction of apoptosis.

Bax expression is involved in 20(S)-GRh2-induced apoptosis. It was found that 20(S)-GRh2 treatment induced Bax upregulation and promoted Bax translocation to the mitochondria (Fig. 7A and B). Bax expression was significantly suppressed when VDAC1 was silenced by siRNA (Fig. 7C and D).
The aforementioned results showed that the separation of HK2 from the mitochondria promoted the translocation of Bax to mitochondria and the release of cytochrome c into the cytoplasm.

We hypothesize that treatment of HeLa cells with 20(S)-GRh2 causes metabolic stress leading to upregulation of VDAC1, the translocation of Bax to the mitochondria and subsequent apoptosis of cancer cells (Fig. 8).

**Discussion**

The present study found that 20(S)-GRh2, a pharmacologically active component of *Panax* ginseng, exerted significant cytotoxicity in HeLa cells. Exposure to 20(S)-GRh2 altered mitochondrial function in HeLa cells, leading to mitochondrial-related apoptosis, confirming that 20(S)-GRh2 induces apoptosis of HeLa cells, as reported previously (25,42). In addition, cell death induced by 20(S)-GRh2 was associated with the regulation of energy metabolism. Treatment with 20(S)-GRh2 significantly inhibited OXPHOS and glycolysis. The dual targeting of mitochondrial and glycolysis pathways by 20(S)-GRh2 may explain why ROS generation, MMP collapse and ATP reduction were observed in HeLa cells treated with 20(S)-GRh2.

In addition, the OCR that corresponds to non-mitochondrial O2 consumption was also slightly affected by 20(S)-GRh2. Mitochondria consume >95% of the O2 of aerobic higher organisms; however, 10% of cellular oxygen uptake in mammal cells is due to non-mitochondrial respiration (43). It has been proposed that an increase in non-mitochondrial oxygen consumption might serve as a protective mechanism to remove ROS when it is present at potentially harmful concentrations (44). This suggestion provides a plausible explanation for the decrease in non-mitochondrial oxygen consumption observed in the present study, as it would help increase in ROS generation and oxidative stress to further induce apoptosis.

The mechanism by which 20(S)-GRh2 regulates cell death and energy metabolism was next elucidated. 20(S)-GRh2 influenced the protein levels of VDAC1 and HK2, which are important regulators of glucose metabolism (45,46), and the changes in VDAC1 and HK2 protein levels were inversely associated. Treatment with 20(S)-GRh2 inhibited mitochondrial metabolism, leading to dissociation of HK2 from mitochondrial VDAC1, which regulated the transfer of Bax to the mitochondria and allowed cytochrome c to enter the cytosol via the outer mitochondrial membrane. These events led to apoptosis induced by metabolic stress. A loss
of the MMP during 20(S)-GRh2-induced apoptosis and the opening of mitochondrial permeability transition pores were observed. The present findings are the first to show that this mechanism is associated with 20(S)-GRh2-induced apoptosis. These results also showed that VdAc1 may be a target in the process of HeLa cell apoptosis induced by 20(S)-GRh2.

By transferring HK2 to the mitochondria, an acidic anti-hypoxic microenvironment suitable for tumor cell survival can be formed and maintained (47). The interaction between HK2 and VdAc1 provides metabolic preponderance for cancer cells by enhancing anaerobic glycolysis, which is called the Warburg effect (48). When HK2 separates from the mitochondria, cells become sensitive to many apoptotic factors (49). Many anticancer compounds that target the mitochondria have been shown to release HK2 from the mitochondria (50-52). The present study found that 20(S)-GRh2 disaggregated HK2 from the mitochondria in HeLa cells.

Figure 5. Upregulation of VdAc1 plays a role in 20(S)-GRh2-induced apoptosis. (A) RT-qPCR was performed to examine VdAc1 expression in HeLa cells. Data were analyzed using one-way ANOVA and presented as the mean ± standard deviation. n=3; *P<0.05 vs. control. (B) Western blot was applied to assess VdAc1 protein expression. Data were analyzed using one-way ANOVA and presented as the mean ± standard deviation. n=3; ***P<0.001 vs. control. (C) VdAc1 expression levels in cells transfected with NC, siVc1-1 or siVc1-2 was assessed by RT-qPCR. Data were analyzed using one-way ANOVA and presented as the mean ± standard deviation. n=3; *P<0.05 vs. NC. (D) VdAc1 protein expression levels in cells transfected with NC, siVc1-1 or siVc1-2 was assessed by western blotting. (E and F) Flow cytometry was performed to assess the apoptosis of HeLa cells transfected with NC or siVc1-2. Data analyzed using one-way ANOVA and presented as the mean ± standard deviation. n=3; ***P<0.001 vs. NC control; **P<0.01 vs. siVc1-2 control. RT-qPCR, reverse transcription-quantitative PCR; 20(S)-GRh2, 20(S)-ginsenoside Rh2; VdAc1, voltage-dependent anion channel 1; siVc1, small interfering RNA targeting VdAc1; NC, negative control.
However, whether desorption of HK2 was due solely to the direct interaction of 20(S)-GRh2 with VDAC1 was unclear, and an interaction between 20(S)-GRh2 and HK2 cannot be ruled out. Thus, 20(S)-GRh2-induced cell death is a cumulative effect of VDAC1 blockade and desorption of HK2 from mitochondria. ROS production, MMP collapse and ATP decrease may be the result of VDAC shutdown. The wide range of antitumor activities of 20(S)-GRh2 includes inhibiting tumor growth, restraining tumor progression and strengthening chemotherapeutic responses (24). Previous studies have shown that 20(S)-GRh2 inhibits the growth of many tumor cell types and its potential mechanism has been confirmed. Ginsenoside Rh2 also inhibited proliferation and promotes apoptosis of H1299 cells by inducing endoplasmic reticulum stress mediated by ROS (53). The synergistic effect of autophagy and β-catenin signal transduction inhibited the proliferation and migration of human liver cancer HepG2, Hep3B and Huh7 cells, and restrained tumor growth in HepG2-xenografted mice (54,55). Ginsenoside Rh2 inhibited cell proliferation, promoted apoptosis and reversed the resistance of colon cancer to the chemotherapeutic drug oxaliplatin (56). The p53 tumor suppressor is also activated in response to ginsenosides (25,57). Ginsenoside Rh2 also induced apoptosis of HcT-116 and SW-480 cells via p53 activation (58).

In conclusion, the present findings have revealed VDAC1 as a new target of 20(S)-GRh2. These results have provided further understanding of the metabolic mechanism of ginsenoside Rh2 against cancer and have validated the potential clinical value for 20(S)-GRh2 or anticancer drugs based on 20(S)-GRh2 in cervical cancer in vitro. However, the present study only studied a human papillomavirus (HPV)-positive cervical cancer cell. Based on evidence suggesting the heterogeneity in cervical cancer-related pathways in terms of HPV status, we are currently examining the effects of 20(S)-GRh2 in a comprehensive panel of human cervical cancer-derived cell lines, including HPV-positive and HPV-negative cell lines. Future research will examine the efficacy of 20(S)-GRh2 in an in vivo system.

Figure 6. 20(S)-GRh2-induced reduction of HK2 results in VDAC1 upregulation. (A) Mitochondrial and (B) cytosolic HK2 expression was determined using western blotting. HeLa cell lysates were analyzed by western blotting with antibodies for (C) HK2 and (D) VDAC1. Data were analyzed using one-way ANOVA and presented as the mean ± standard deviation. n=3. *P<0.05 vs. control. 20(S)-GRh2, 20(S)-ginsenoside Rh2; VDAC1, voltage-dependent anion channel 1; HK2, hexokinase 2.
Figure 7. Mitochondrial translocation of Bax is involved in VDAC1-mediated apoptosis, which is induced by 20(S)-GRh2. (A and B) Subcellular localization of Bax was examined by western blotting. Prohibitin acted as the loading control for mitochondrial samples; β-actin acted as the loading control for cytosolic fractions. Data analyzed using one-way ANOVA and presented as the mean ± standard deviation. n=3. *P<0.05 and ***P<0.001 vs. control (mitochondrial fraction). #P<0.05 and ##P<0.01 vs. control (cytosol fraction). (C and D) Western blot analysis showed that Bax expression is downregulated in VDAC1-silenced HeLa cells. Data were analyzed using one-way ANOVA and presented as the mean ± standard deviation. n=3. *P<0.05 and ***P<0.001 vs. NC control. #P<0.05 and ##P<0.01 vs. siVC1 control. 20(S)-GRh2, 20(S)-ginsenoside Rh2; siVC1, small interfering RNA targeting voltage-dependent anion channel 1; NC, negative control.

Figure 8. 20(S)-GRh2 causes metabolic stress leading to upregulation of VDAC1, translocation of Bax to mitochondria and subsequent apoptosis of HeLa cells. 20(S)-GRh2, 20(S)-ginsenoside Rh2; VDAC1, voltage-dependent anion channel 1; HK2, hexokinase 2; mito, mitochondria; casp, caspase.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ML and JW managed data collection and designed the experiments. YL, QJ, SL and SW performed the experiments. YL and ML wrote the manuscript. DZ and XB contributed to data analysis, supervised the experiments and helped draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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