20(S)-Ginsenoside Rg3-induced apoptosis in HT-29 colon cancer cells is associated with AMPK signaling pathway

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Abstract. 20(S)-ginsenoside Rg3 [20(S)-Rg3)], one of the main constituents isolated from *Panax ginseng*, has been shown to have an anti-cancer effect and to induce apoptosis by interfering with several signaling pathways. However, the molecular mechanisms of AMP-activated protein kinase (AMPK) associated with apoptosis in HT-29 colon cancer cells remain unclear. In the present study, we investigated whether 20(S)-Rg3 exerts an anti-proliferative effect and induces apoptosis by modulating the AMPK signaling pathway in HT-29 cells. 20(S)-Rg3-treated cells displayed several apoptotic features, including DNA fragmentation, proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) and morphological changes. 20(S)-Rg3 down-regulated the expression of anti-apoptotic protein B-cell CLL/lymphoma 2 (Bcl2), up-regulated the expression of pro-apoptotic protein of p53 and Bcl-2-associated X protein (Bax), and caused the release of mitochondrial cytochrome *c*, PARP, caspase-9 and caspase-3. However, 20(S)-Rg3-induced apoptosis was completely abolished in the presence of compound C (AMPK inhibitor) or small interfering RNA for AMPK (siAMPK). In addition, STO-609 (CaMKKβ inhibitor) attenuated 20(S)-Rg3-induced AMPK activation and apoptosis. These results suggest that 20(S)-Rg3-induced apoptosis in HT-29 cells is mediated via the AMPK signaling pathway, and that 20(S)-Rg3 is capable of inducing apoptosis in colon cancer.

Introduction

Colorectal cancer is one of the leading causes of cancer-related death in Western countries, and its incidence is increasing rapidly in Asia (1,2). Surgical resection followed by adjuvant chemotherapy has been considered the optimal treatment approach for patients with colon cancer (3,4). However, the approach has a high relapse rate after surgery, and anti-cancer drugs have many detrimental side effects (5). To develop an anti-cancer agent with improved efficacy and minimal toxicity, cancer chemoprevention with medicinal plants has been thoroughly investigated. Numerous reports have documented that anti-cancer components derived from medicinal plants play a partial role in the prevention or therapy of various types of human cancer. These components have been shown to inhibit carcinogenesis by inhibiting cell proliferation and inducing apoptosis (2,6).

Ginseng is a medicinal herb commonly used in Asian countries for numerous purposes (7,8). It has been reported to have anti-proliferative properties in several types of cancer, including ovarian, breast and lung cancer, and melanoma (9,10). Steamed *Panax ginseng* (red ginseng) is the preferred choice over unsteamed white ginseng (11), as studies have shown that red ginseng has a greater anti-proliferative benefit than white ginseng, most likely due to the differences in their ginsenoside profiles (12). 20(S)-ginsenoside Rg3 (Fig. 1), one of the main constituents found in red ginseng, has been shown to exert inhibitory effects on various cancers through inductive effects on several signaling pathways (13-15). However, it remains to be determined whether apoptotic cell death is associated with the AMP-activated protein kinase (AMPK) signaling pathway in HT-29 colon cancer cells treated with 20(S)-Rg3.

AMPK is a member of a serine/threonine protein kinase and plays an essential role as an energy sensor in eukaryotic cells (16). Recently, AMPK was shown to be involved in cellular homeostasis and emerged as a pivot point between cell survival and apoptosis (16-18). Previous studies have reported that AMPK activation regulates apoptosis in cancer cells via a signaling pathway that includes the up-regulation of p53 protein, activation of caspases, generation of ROS and inhibition of the proteolytic cleavage of poly (ADP-ribose) polymerase (PARP)-LKB1 (17-20).

In the present study, the anti-proliferative activity of 20(S)-Rg3 was determined and its effect was shown to be related to the induction of apoptosis in HT-29 colon cancer cells. The mechanisms of apoptosis induction by 20(S)-Rg3 were explored with respect to the AMPK signaling pathway.

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Materials and methods

Reagents. RPMI-1640, FBS and penicillin/streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). Compound C was from Calbiochem (San Diego, CA, USA) and the Annexin V-FITC apoptosis detection kit was purchased from BD Bioscience (San Diego, CA, USA). Antibodies against phospho-AMPK, AMPK, phospho-ACC, ACC, caspase-3, caspase-9, p53, PARP and CaMKKβ were from Cell Signaling Technology (Beverly, MA, USA), and Bcl2, Bax, cytochrome *c* and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein extraction and ECL-reagent kits were from Intron Biotechnology Inc. (Beverly, MA, USA). 20(S)-Rg3 was obtained from the Central Research Center, ILHWA Pharmaceutical Co. (Guri, Korea).

Cell culture and cell survival assay. The HT-29 colon cancer cell line was purchased from the American Type Culture Collection (Rockville, MD, USA), cultured in RPMI-1640 containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37˚C in a humidified atmosphere of 5% CO_2 in air. The effects of the 20(S)-Rg3 on HT-29 cell growth, expressed as the percentage of cell survival, were determined using the MTS assay. The cells were seeded on 96-well plates at $3x10^4$ cells/well and treated with various concentrations of 20(S)-Rg3 (0, 10, 50, 100 and 200 μM) for 24 or 48 h. Cell viability was detected by the CellTiter 96® AQueous One solution Cell Proliferation Assay (Promega, Madison, WI, USA).

Annexin V-FITC/propidium iodide flow cytometric analysis. Phosphatidylserine on the outside of the apoptoic cells was determined using the Annexin V-FITC apoptosis detection kit. Briefly, HT-29 cells ($9x10^5$ cells /well) were treated with various concentrations of 20(S)-Rg3 (0, 10, 50 and 100 μM) for 24 h. The cells were collected and resuspended in 100 μl of 1X Annexin binding buffer, then 5 μl of conjugated Annexin V-FITC and 10 μl of propidium iodide (PI) buffer were added prior to flow cytometric analysis. Cells were excited at 488 nm and the emission measured at 525 nm and 610 nm for Annexin V-FITC and PI fluorescence, respectively. The cells were analyzed using flow cytometry (Beckman, San Diego, CA, USA).

DNA ladder assay. After treatment with 20(S)-Rg3, the HT-29 cells were lysed in buffer containing 10 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA, 0.5% Triton X-100 and 20% SDS. Proteinase K (10 mg/ml) was added and the mixture was incubated for 4 h at 55˚C, then extracted with phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated with 2 volumes of ice-cold absolute ethanol. After extraction with phenol buffer (phenol-chloroform-isoamyl alcohol), the pellets were incubated with TE buffer (10 mM Tris-HCl, 1 mM EDTA) and RNase (2 mg/ml) for 1 h at 37˚C. DNA fragmentation was determined by electrophoresis on 2% agarose gel containing ethidium bromide, and photographed using the I-MAX Gel Image analysis system (Core-Bio, Seoul, Korea).

Preparation of whole cell lysates, cytosolic and mitochondrial fractions. For preparation of whole cell lysates, the cells were collected, washed twice with ice-cold PBS and lysed using a protein extraction kit. Insoluble protein was removed by centrifugation at 13,000 g for 15 min. For the preparation of cytosolic and mitochondrial protein, cells were collected and washed twice with ice-cold PBS, then resuspended in ice-cold cell extraction buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 μg/ml pepstatin A and leupeptin) containing 250 mM sucrose for 30 min on ice. The cells were then homogenized and subjected to centrifugation for 10 min at 1,000 g to remove unbroken cells, pellet nuclei and heavy membranes. The postnuclear supernatant was further centrifuged for 30 min at 13,000 g, and the mitochondria-enriched heavy membrane (pellet) and cytosolic fractions (supernatant) were obtained. The supernatant was collected and stored at 70˚C for Western blot analysis. The mitochondria rich fraction was solubilized using a protein extraction kit and then centrifuged at 13,000 g for 15 min. The supernatant was collected and stored at 70˚C for Western blot analysis.

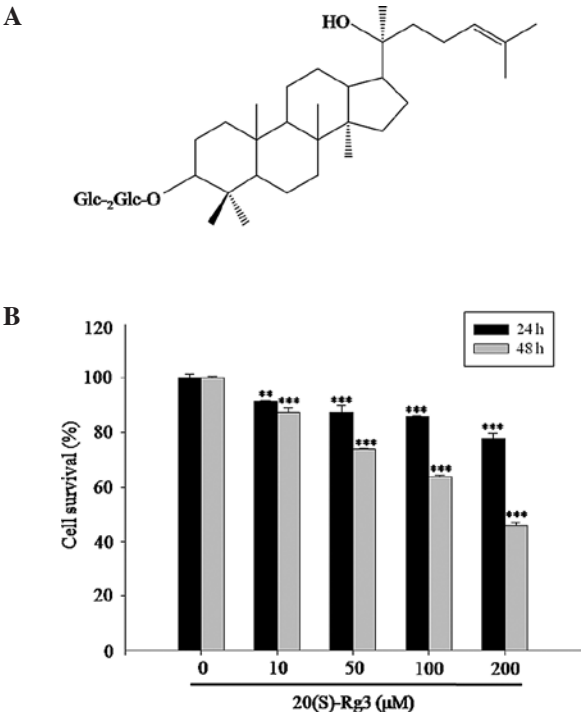

Figure 1. (A) Chemical structure of 20(S)-Rg3. (B) Effect of 20(S)-Rg3 on the cell viability of HT-29 cells. Data are expressed as the mean ± SEM of triplicate determinations. $^{**}p<0.01$ and $^{***}p<0.001$, significantly different compared to the control, as determined by the Student-Newman-Keul test.

RNA interference. To knock down endogenous AMPKα, HT-29 cells were transiently transfected with 10 nM of chemically synthesized siRNA targeting AMPKα 1/2 or with non-silencing siRNA using a siRNA transfection reagent according to the manufacturer's recommendations. The transfected cells were used for Western blot analysis.

Immunoprecipitation. Cell lysates (300 μg) were pre-cleared with protein A/G Sepharose beads and incubated with CaMKKβ antibody for 4 h at 4˚C. The reaction mixture was then combined with protein-A/G Sepharose beads and incu-

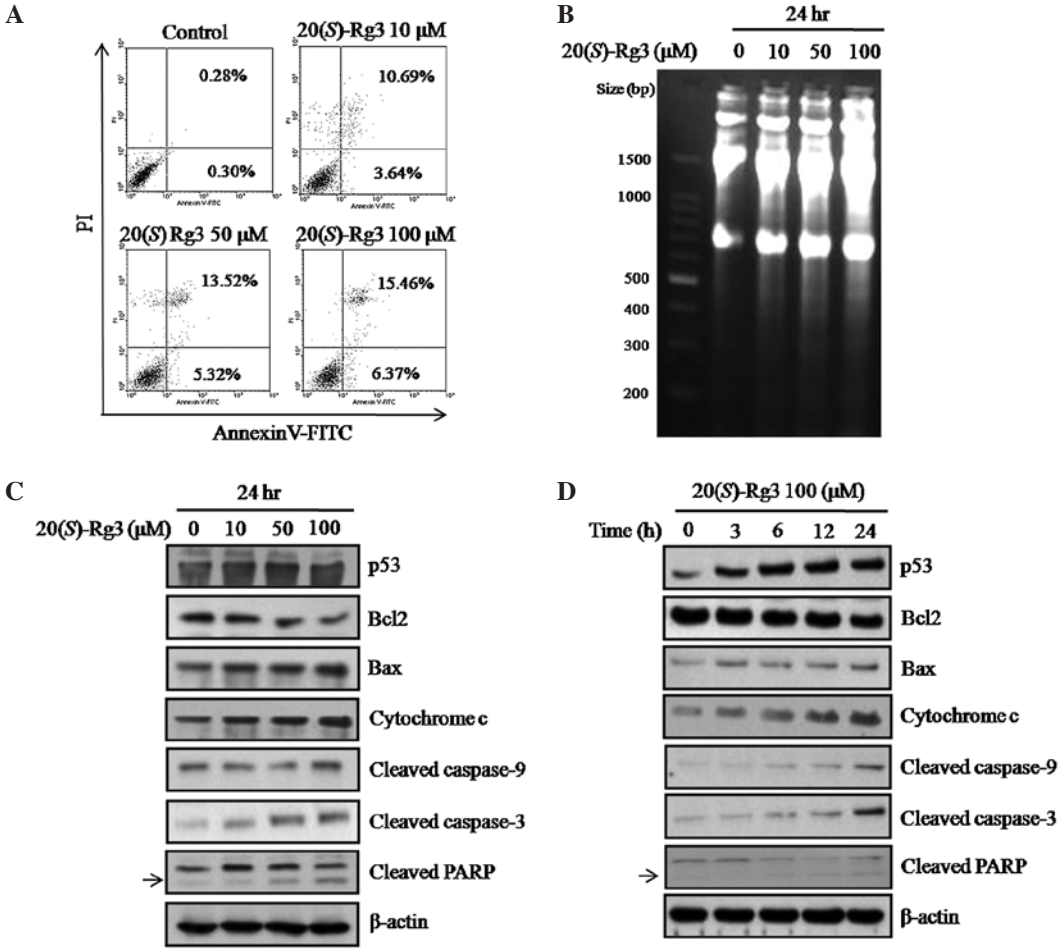

Figure 2. 20(S)-Rg3 induces apoptosis in HT-29 cells. (A) Detection of apoptotic cells by Annexin V and PI double staining. Cells were incubated using the indicated concentrations of 20(S)-Rg3 for 24 h, stained with Annexin V and PI labeling, and analyzed using flow cytometery. (B) DNA fragmentation in HT-29 cells exposed to the indicated concentrations of 20(S)-Rg3 for 24 h. The isolated DNA was loaded into a well on a 2% agarose gel and electrophoresed. Determination of apoptotic proteins in HT-29 cells exposed to the indicated concentrations of 20(S)-Rg3 for 24 h (C) or treated with 100 μM of 20(S)-Rg3 for the times indicated (D). Equal amounts of cell lysate (40 μg of extract) were resolved by SDS-PAGE and analyzed by Western blotting, and the blot was re-probed with anti-actin antibody to confirm equal protein loading.

bated for 2 h at 4˚C. Following centrifugation at 1,000 g for 1 min, the precipitate was collected and washed twice with PBS, then 1X SDS buffer was added. After boiling for 1 min, the supernatant was collected. The phosphorylated form of CaMKKβ was detected using Western blot analysis.

Western blot analysis. Equal amounts of protein (40 μg) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with appropriate primary antibodies (anti-AMPK, anti-pAMPK, anti-ACC, anti-pACC, anti-p53, anti-Bax, anti-Bcl2, anti-cytochrome *c*, anti-PARP, anti-caspase-3 and -9 and anti-actin) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-rabbit IgG (Santa Cruz Biotechnology) secondary antibodies. Reactive bands were visualized with an enhanced chemiluminescence solution (Amersham, Sweden).

Statistical analysis. Data were presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by the Student-Newman-Keuls test. A value of $p<0.05$ was considered to be significant.

Results

20(S)-Rg3 inhibited cell proliferation and induced apoptosis. To explore the mechanism by which 20(S)-Rg3 exerts its effects against colon cancer cells, its effects on cell proliferation were examined first. HT-29 cells ($3x10^4$ cell/well) were exposed to various concentrations (0, 10, 50, 100 and 200 μM) of 20(S)-Rg3 for 24 h or 48 h. 20(S)-Rg3 showed significant anti-proliferative effects in a dose- and time-dependent manner (Fig. 1B). As the colon cancer cells exhibited sensitivity to 20(S)-Rg3 in terms of proliferation, the effect of 20(S)-Rg3 on the level of cellular apoptosis was further examined. HT-29 cells were exposed to various concentrations (0, 10, 50 and 100 μM) of 20(S)-Rg3 for 24 h, and the effect of 20(S)-Rg3-induced apoptosis was determined using flow cytometry. As shown in Fig. 2A, 20(S)-Rg3 induced apoptosis in a concentration-dependent manner. The percentages of cells in early apoptosis were displayed as 3.64, 5.32 and 6.37% at 10, 50 and 100 μM of 20(S)-Rg3, respectively (lower right quadrant). Moreover, late-apoptotic cells were displayed as 10.69, 13.52 and 15.46% at 10, 50 and 100 μM of 20(S)-Rg3 (upper right quadrant). Apoptosis was examined by a DNA ladder assay.

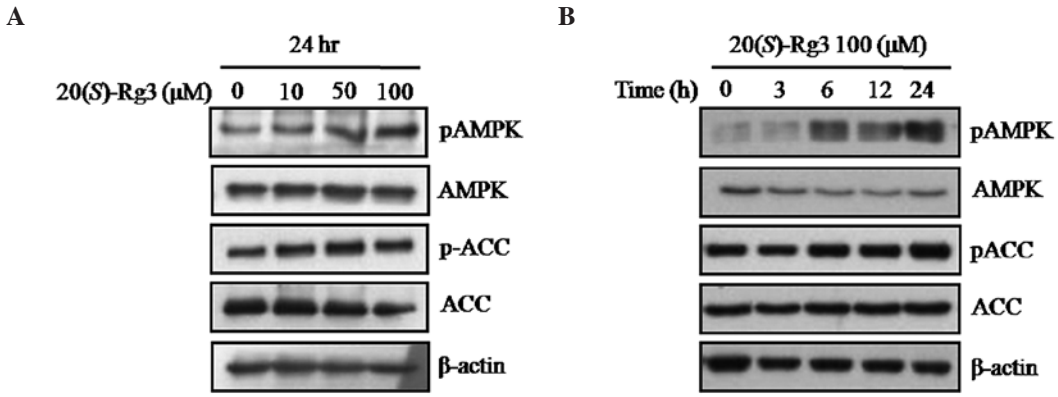

Figure 3. Effect of 20(S)-Rg3 on AMPK and ACC phosphorylation in HT-29 human colon cancer cells. The cells were exposed to the indicated concentrations of 20(S)-Rg3 for 24 h (A) or treated with 100 μM of 20(S)-Rg3 for the times indicated (B). Equal amounts of cell lysate (40 μg of extract) were resolved by SDS-PAGE and analyzed by Western blotting, and the blot was re-probed with anti-actin antibody to confirm equal protein loading.

After treating the HT-29 cells with various concentrations of 20(S)-Rg3 for 24 h, typical DNA ladders were clearly visible in Et-Br stained gels (Fig. 2B).

Expression of 20(S)-Rg3-induced apoptosis-associated proteins. To elucidate the molecular mechanisms of 20(S)-Rg3-induced apoptosis in HT-29 cells, the expression of several apoptosis-associated proteins was examined. HT-29 cells were exposed to various concentrations (0, 10, 50 and 100 μM) of 20(S)-Rg3 for 24 h (Fig. 2C) or treated with 100 μM of 20(S)-Rg3 for up to 24 h (Fig. 2D). As shown in Fig. 2C and D, 20(S)-Rg3 induced a decrease in the protein level of Bcl2 and an increase in the level of Bax in a concentration- and time-dependent manner. A densitometric analysis of the bands revealed that 20(S)-Rg3 treatment resulted in a dose-dependent increase in the Bax/Bcl2 ratio (data not shown). p53 protein expression was increased in a dose- and time-dependent manner. To monitor the release of mitochondrial protein into the cytosol, the cytosolic and mitochondrial fractions were separated and the release of cytochrome *c* into the cytosol was monitored. As shown in Fig. 2C and D, treatment of HT-29 cells with 20(S)-Rg3 resulted in the release of cytochrome *c* into the cytoplasm in a concentration- and time-dependent manner. 20(S)-Rg3 treatment was also found to cause a significant increase in the active forms of caspase-9 and -3 and PARP.

20(S)-Rg3 stimulated AMPK phosphorylation. To investigate the effect of 20(S)-Rg3 on AMPK phorsphorylation, HT-29 cells were exposed to various concentrations (0, 10, 50 and 100 μM) of 20(S)-Rg3 for 24 h or treated with 100 μM of 20(S)-Rg3 for up to 24 h. As shown in Fig. 3A and B, 20(S)-Rg3 markedly activated AMPK in a concentration- and time-dependent manner. Consistent with the increase in AMPK activity, the phorsphorylation of ACC, which is an immediate substrate for AMPK, was also increased in a concentration- and time-dependent manner.

20(S)-Rg3 induced apoptosis by activating AMPK. To verify the hypothesis that the apoptosis-inducing activity of 20(S)-Rg3 is associated with the AMPK pathway, a synthetic AMPK inhibitor (compound C) or small interfering RNA for AMPK were employed. In the presence of compound C (10 or 20 μM), the 20(S)-Rg3-induced phorsphorylation of AMPK and ACC was significantly attenuated (Fig. 4B). Next, to determine whether AMPK activation by 20(S)-Rg3 was associated with the induction of apoptosis in HT-29 cells, FACS analysis was performed in order to quantify the rate of cell apoptosis using double staining of Annexin V-FITC and PI. As shown in Fig. 4A, 20(S)-Rg3-induced apoptosis was markedly abrogated by compound C. Next, the expression of apoptotic proteins was determined in the presence of compound C. As shown in Fig. 4C, the 20(S)-Rg3-induced decrease in mitochondrial Bcl2 protein expression was reversed by pre-treatment with compound C. By contrast, 20(S)-Rg3-induced increases in p53, mitochondrial Bax, cytosolic cytochrome *c* and cleaved caspase-9 and -3 protein expression were significantly decreased by pre-treatment with compound C. To confirm the effect of compound C on 20(S)-Rg3-induced apoptosis, siRNA for AMPK was incubated with cells for 48 h, and then the cells were treated with 20(S)-Rg3 for 24 h. As shown in Fig. 5A, HT-29 cells transfected with siRNA for AMPK had decreased 20(S)-Rg3-induced AMPK and ACC expression, as compared to the control cells. As expected, siRNA for AMPK reversed 20(S)-Rg3-induced decreases in mitochondrial Bcl2 protein expression and increases in p53, mitochondrial Bax, cytosolic cytochrome *c* and cleaved caspase -9 and -3 protein expression. These results demonstrated that 20(S)-Rg3-induced apoptosis occurred via the AMPK pathway in HT-29 cells.

CaMKKβ is an upstream kinase for AMPK. In order to further understand the signaling pathway involved in 20(S)-Rg3-mediated signaling, HT-29 cells were treated with 100 μM of 20(S)-Rg3 for up to 24 h, then the protein expression of p-CaMKKβ was analyzed using Western blot analysis. As shown in Fig. 6A, p-CaMKKβ protein expression was markedly increased at 24 h when the cells were treated with 100 μM of 20(S)-Rg3. Next, to determine whether the AMPK activation is caused by CaMKKβ, HT-29 cells were treated with STO-609, a CaMKKβ inhibitor. Pre-treatment of HT-29 cells with STO-609 significantly attenuated 20(S)-Rg3-stimulated phosphorylation of CaMKKβ in a dose-dependent manner (Fig. 6B), and inhibited the phosphorylation of AMPK and ACC (Fig. 6C).

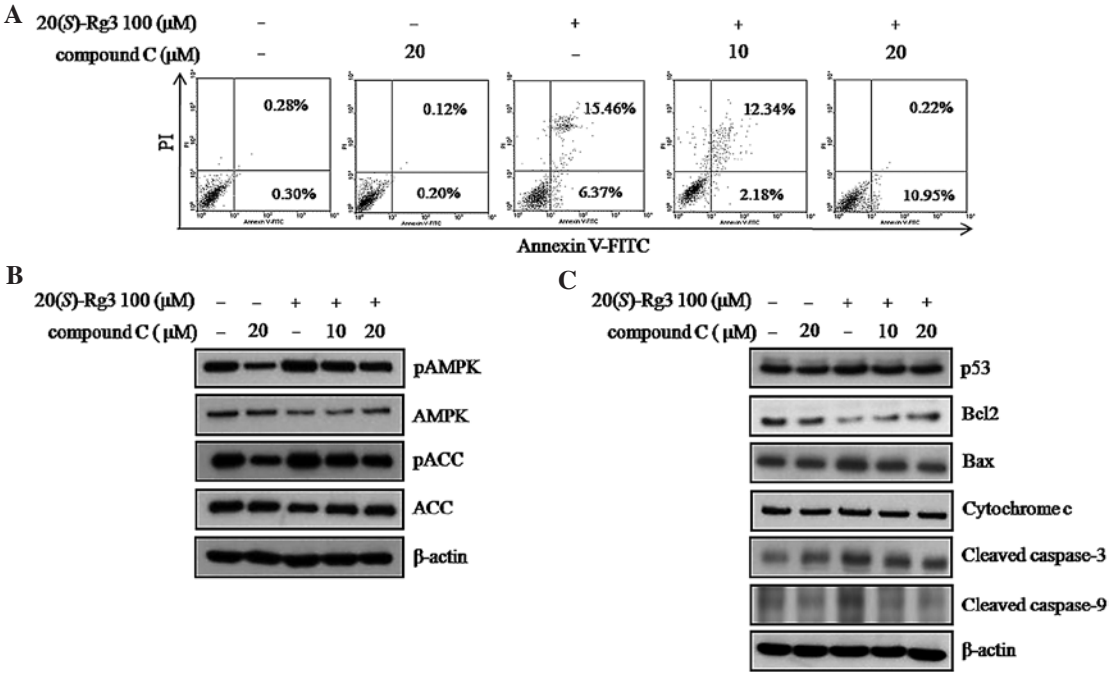

Figure 4. Effects of compound C on (A) 20(S)-Rg3 induced apoptosis, (B) AMPK phosphorylation and (C) expression of apoptosis proteins in HT-29 cells. The cells were pretreated with compound C for 2 h, and treated with 20(S)-Rg3 for 24 h. Detection of apoptotic cells was performed using Annexin V and PI double staining. The phosphorylation of AMPK and ACC, and the expression of apoptosis-related signal molecules were determined by Western blotting. β-actin was used as an internal control to evaluate relative protein expression.

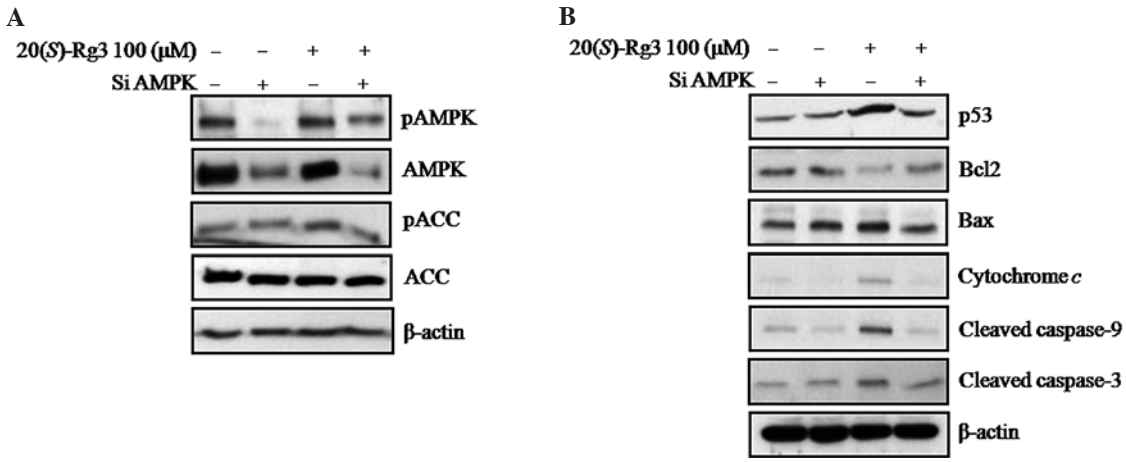

Figure 5. Effects of siRNA for AMPK on (A) 20(S)-Rg3-induced AMPK phorsphorylation and (B) expression of apoptosis-related signal proteins in HT-29 cells. The cells were transfected with AMPK siRNA for 48 h, and exposed to 100 μM 20(S)-Rg3 for 24 h. Cell lysates were used for Western blot analysis.

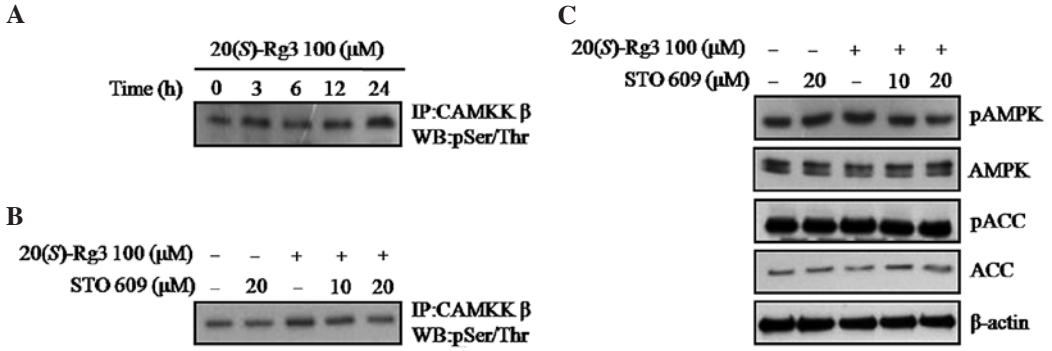

Figure 6. CaMKKβ is an upstream kinase for 20(S)-Rg3-induced AMPK phorsphorylation in HT-29 cells. The cells were treated with (A) 100 μM 20(S)-Rg3 for the times indicated and (B) STO-609 for 2 h prior to stimulation with 100 μM 20(S)-Rg3, then immunoprecipitated with an antibody against CaMKKβ and Thr172. CaMKKβ and Thr172 phosphorylation was determined by Western blotting. (C) pAMPK and pACC expression in the presence of STO-609 was also determined by Western blotting.

Discussion

20(S)-Rg3, one of the main components found in red ginseng, has been shown to inhibit cancer cell proliferation, invasion and metastasis and to induce differentiation and apoptosis (13-15,21,22). As previously reported, the mechanisms responsible for 20(S)-Rg3-induced apoptosis appear to be varied, including NF-κB and activator protein (AP-1) activation, inhibition of angiogenesis and activation of caspase-3 (13-15). Nevertheless, the cellular and molecular mechanisms underlying 20(S)-Rg3-induced apoptosis associated with AMPK signaling pathway in HT-29 cells have not been defined. The present study demonstrated for the first time that 20(S)-Rg3 induced the apoptosis of HT-29 cancer cells via the CaMKKβ/AMPK signaling pathway.

Apoptosis is an important mechanism of 20(S)-Rg3-induced cell death, and susceptibility to the apoptosis of cancer cells is an important determinant of the efficacy of chemotherapy. Our results showed that 20(S)-Rg3 significantly decreased the number of viable cell in a concentration-dependent manner (Fig. 1B). Apoptotic cells induced by 20(S)-Rg3 displayed DNA fragmentation (Fig. 2B). Annexin-V FITC/PI FACS analysis further confirmed the results of a DNA ladder assay by showing that the membrane alterations related to apoptosis and the percentage of apoptosis were increased in a concentration-dependent manner (Fig. 2B and C). These results suggest that 20(S)-Rg3 markedly inhibited cell proliferation and induced apoptosis in HT-29 colon cancer cells.

Numerous studies have indicated that the apoptotic process in cancer cells occurs via two major pathways. The first pathway operates via death receptors on the cell surface, such as Fas and other members of the tumor necrosis factor (TNF) receptor family, which activates caspase-8. Caspase-8 drives its activation through self-cleavage, and subsequently activates downstream caspases such as caspase-9 and -3 (23). The second pathway involves a mitochondrial pathway that causes the release of cytochrome *c* into the cytosol, which triggers apoptosis by activation of caspase-9 in the presence of apoptosis protease activation factor-1 (Apaf-1). This in turn results in the activation of downstream caspase-3, which cleaves PARP (24,25). Recent data indicate that the mitochondrial pathway is controlled and regulated by Bcl-2 family protein (5,22-25). Bcl-2 family proteins such as Bcl-2 and Bax have been identified as major regulators in controlling the release of mitochondrial cytochrome *c* (26). Moreover, p53-mediated apoptosis is associated with Bcl-2 and Bax (27,28). Our data indicate that there was an increase in Bax expression and a decrease in Bcl2 expression, which resulted in a reduction of mitochondrial membrane potential and increased permeability of the mitochondrial outer membrane to cytochrome w*c*. Released cytochrome *c* binds to Apaf-1 and activates procaspase-9. Activation of caspase-9 results in the activation of caspase-3, -6 and -7, which function as downstream effectors of the cell death program. Caspase-3 is an executioner caspase activated by either a mitochondrial pathway involving caspase-9 or a death receptor pathway involving caspase-8 (23-25). According to the results shown in Fig. 2C and D, 20(S)-Rg3 induced apoptosis in HT-29 colon cancer cells via the mitochondrial pathway (caspase-9). However, a death receptor pathway cannot be excluded, since procaspase-8 was also cleaved in a concentration-dependent manner when the cells were treated with 20(S)-Rg3 (data not shown).

AMPK is a conserved serine/threonine protein kinase regulator of cellular metabolism (29). It has been implicated in various important aspects of cell biology, including the stimulation of angiogenesis, regulation of nitric oxide synthesis and apoptosis (30-33). In the present study, we examined whether 20(S)-Rg3-induced apoptosis is associated with AMPK activation. Our results show that 20(S)-Rg3 stimulated AMPK activation in HT-29 colon cancer cells (Fig. 3A and B), and that 20(S)-Rg3-induced apoptosis was completely inhibited by both AMPK inhibitor (compound C) or siAMPK (Figs. 4 and 5). These results suggest that 20(S)-Rg3-induced AMPK activation may be responsible for 20(S)-Rg3-induced apoptosis, and that it serves as a positive regulator of apoptosis. Currently, LKB1 and CaMKK are the known upstream kinases of AMPK (34). To identify upstream kinases for AMPK, we examined whether 20(S)-Rg3 is capable of activating LKB1 and CaMKK in HT-29 cells. As shown in Fig. 6, 20(S)-Rg3 phosphorylated CaMKKβ in a time-dependent manner, and 20(S)-Rg3-induced phosphorylation of CaMKKβ and AMPK was inhibited in the presence of a CaMKK inhibitor, STO-609. Of note, LKB1 was also activated by 100 μM of 20(S)-Rg3 in a time-dependent fashion (data not shown). Therefore, LKB1 and CaMKK both were considered as upstream kinases for AMPK in 20(S)-Rg3-induced apoptotic event of HT-29 cells.

In conclusion, we examined the pathway involved in 20(S)-Rg3-induced apotosis in HT-29 colon cancer cells, and found that 20(S)-Rg3-induced apoptosis mainly requires the mitochondrial pathway involving Bax/cytochrome *c*/caspase-9, and that this apoptosis occurred via the activation of the AMPK pathway. These findings suggest that the AMPK signaling pathway is associated with apoptosis induction by 20(S)-Rg3 in HT-29 colon cancer cells, and is worth exploring as a potential target for colon cancer.

Acknowledgements

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