



Research article

AMP-activated protein kinase determines apoptotic sensitivity of cancer cells to ginsenoside-Rh2

Min-Jung Kim¹, Hee Yun¹, Dong-Hyun Kim², Insug Kang¹, Wonchae Choe¹, Sung-Soo Kim¹, Joohun Ha^{1,*}¹ Department of Biochemistry and Molecular Biology, Medical Research Center and Biomedical Science Institute, School of Medicine, Kyung Hee University, Seoul, Korea² Department of Life and Nanopharmaceutical Sciences, College of Pharmacy, Kyung Hee University, Seoul, Korea

ARTICLE INFO

ABSTRACT

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Received in Revised form
12 September 2013
Accepted 13 September 2013

Keywords:

AMP-activated protein kinase
apoptosis
cancer
p38 MAPK
Panax ginseng

regard to the control of many diseases, including cancer. In this study, the anticancer effect of ginsenosides on human cancer cells was investigated and compared. Among the tested compounds, ginsenoside-Rh2 displays the highest inhibitory effect on cell viability in HepG2 cells. Ginsenoside-Rh2, a ginseng saponin isolated from the root of *Panax ginseng*, has been suggested to have potential as an anticancer agent, but the underlying mechanisms remain elusive. In the present study, we have shown that cancer cells have differential sensitivity to ginsenoside-Rh2-induced apoptosis, raising questions regarding the specific mechanisms responsible for the discrepant sensitivity to ginsenoside-Rh2. In this study, we demonstrate that AMP-activated protein kinase (AMPK) is a survival factor under ginsenoside-Rh2 treatment in cancer cells. Cancer cells with acute responsiveness of AMPK display a relative resistance to ginsenoside-Rh2, but cotreatment with AMPK inhibitor resulted in a marked increase of ginsenoside-Rh2-induced apoptosis. We also observed that p38 MAPK (mitogen-activated protein kinase) acts as another survival factor under ginsenoside-Rh2 treatment, but there was no signaling crosstalk between AMPK and p38 MAPK, suggesting that combination with inhibitor of AMPK or p38 MAPK can augment the anticancer potential of ginsenoside Rh2.

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1. Introduction

Ginseng saponins have various pharmacological effects with regard to the modulation of the progression of many diseases, including cancer, diabetes, immune disorders, and neurodegenerative disease [1]. Ginseng might mediate its antidiabetic action through a variety of mechanisms, including modulation of insulin secretion [2], regulation of apigenic transcription factor PPAR- γ [3], and control of glucose level [4] and glucose transport [5]. There have also been many reports describing the immunomodulating effects of ginseng. Ginseng extracts modulate cytokine production [6], enhance CD4(+) T cell activities [7], and restore T lymphocytes function [8]. In addition, ginseng saponins have anticarcinogenic effects through diverse mechanisms, including cell cytotoxicity [9,10], antitumor promotion related to antimetastasis [11] and the inhibition of angiogenesis, synergistic effects in combination with

chemical therapeutic agents [12], and reducing multidrug resistance [13]. Although many ginsenosides have been reported to show anticarcinogenic effects, there is no report focusing on the comparison of the cytotoxic effects of ginsenosides in various cancer cells.

The major active components of ginseng are ginseng saponins, ginsenosides. Recently, ginsenoside-Rh2 (Fig. 1), a plant glycoside with a dammarane skeleton, has been shown to induce apoptosis in a caspase 3,8-dependent manner [14] or the activation of cyclin A-Cdk2 by caspase 3-mediated cleavage of p21(WAF1/CIP1) [15]. Also, ginsenoside-Rh2 was shown to inhibit proliferation by inducing the protein expression of p21 and reducing the protein levels of cyclin D, which resulted in the downregulation of cyclin/Cdk complex kinase activity, a reduction in phosphorylation of pRb, and the inhibition of E2F release [16] or modulation of MAP kinases [17] in various cancer cells; however, their mechanisms have not yet been clearly elucidated.

* Corresponding author. Department of Biochemistry and Molecular Biology, Medical Research Center and Biomedical Science Institute, School of Medicine, Kyung Hee University, Seoul 130-701, Korea.

E-mail address: hajh@khu.ac.kr (J. Ha).

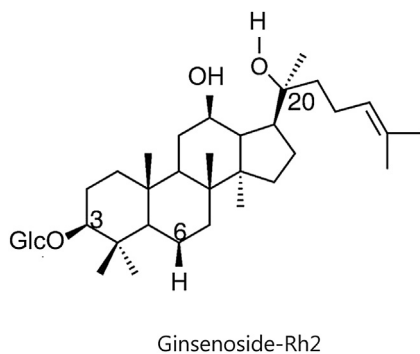


Fig. 1. Chemical structure of ginsenoside-Rh2.

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase that consists of a catalytic α subunit and regulatory β and γ subunits, each of which has at least two isoforms. The activation of AMPK occurs by binding of AMP to the γ subunit, and phosphorylation of Thr172 in the activation loop of the α catalytic subunit by upstream kinases, such as LKB1 and calmodulin-dependent protein kinase kinase (CaMKK) [18]. AMPK is activated under ATP-depleting stresses such as glucose deprivation, hypoxia, and ischemia, and plays a pivotal role in energy homeostasis. Recent studies indicate that AMPK plays a role in linking metabolic syndrome and cancer [19,20]. The AMPK signaling network contains a number of tumor suppressor genes, including LKB1, p53, and TSC2. The tumor suppressor LKB1 has been identified as an upstream activator of AMPK, and other tumor suppressors—p53 and TSC2—are direct substrates of AMPK [20]. In addition to causing cell death, AMPK activation can protect cancer cells against apoptosis in several cases. For example, AMPK activation diminishes apoptosis exposed to anticancer drugs in human gastric carcinoma [21] and glucose deprivation in pancreas cancer cells [22]. Thus, AMPK has pleiotropic functions in regulating cell proliferation and apoptosis, and it is possible that AMPK might be a future target for therapy or prevention of the metabolic syndrome and some cancers.

In this study, we examined the effect of six ginsenosides on cell growth inhibition of the human hepatoma cell line HepG2. Among them, ginsenoside-Rh2 showed the most potent ability to inhibit the growth of cancer cells. Here, we show that some cancer cells have varying sensitivities to ginsenoside-Rh2-induced apoptosis, raising questions concerning the mechanism of inconsistent responses to ginsenoside-Rh2. We discovered that the degree of ginsenoside-Rh2-induced AMPK activation correlates with differences in sensitivity to apoptosis in cancer cell lines. We also observed that p38 MAPK (mitogen-activated protein kinase) acts as a survival factor under ginsenoside-Rh2 treatment, but there was no crosstalk between AMPK and p38 MAPK.

2. Materials and methods

2.1. Cell culture and materials

HepG2, HeLa, DU145, and HCT116 cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics at 37°C with 95% air and 5% CO₂. RPMI Medium 1640 and FBS were purchased from Life Technologies (Grand Island, NY, USA). Compound C was a generous gift from Merck (Darmstadt, Germany). SP600125 and SB203580 were obtained from TOCRIS (Ellisville, MO, USA). 2,7-Dichlorofluorescein (DCFH), N-acetylcysteine (NAC), PD98059, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Hoechst 33342 were

obtained from Sigma-Aldrich (St. Louis, MO, USA). The antibodies that recognize a phosphoactivated form of AMPK-Thr172, and phosphoactivated and total form ACC (Ser79), extracellular signal-regulated kinase (ERK)1 and 2 (Thr202/Tyr204), c-Jun NH₂-terminal kinase (JNK; Thr183/Tyr185), and p38 (Thr180/Tyr182) were from Cell Signaling Technology (Boston, MA, USA). The antibody for poly(ADP-ribose) polymerase (PARP) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The AMPK α antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Ginsenoside-Rc, Rd, Re, Rg3, Rh1, and Rh2 were isolated using a previously described method [23].

2.2. Protein extract and Western blot analysis

HepG2, HeLa, DU154, and HCT116 cells were grown in six-well plates and were washed with cold phosphate-buffered saline (PBS), and lysis buffer (50 mM Tris-HCl at pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 1 μ g/mL leupeptin, 1 μ g/mL aprotinin, and 1 μ g/mL pepstatin; Sigma-Aldrich) was then added to the cells. The plate was gently shaken on ice for 3 min, and the buffer was collected for Western blot analysis. Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose membranes. The membranes were blocked, incubated with primary antibody, washed, and incubated with the secondary HRP-conjugated antibody. The bands were visualized with ECL (Enhanced Chemiluminescence) (Amersham Biosciences, Piscataway, NJ, USA).

2.3. MTT assay

Cells seeded on 96-well microplates at 4,000 per well were incubated with the test compounds for the indicated times. After treatment, media were removed and cells were then incubated with 100 μ L MTT solution (2 mg/mL MTT in PBS) for 4 h. Absorbance was determined using an autoreader.

2.4. Chromatin staining with Hoechst 33342

Apoptosis was observed by chromatin staining with Hoechst 33342. Cells were incubated with each stimulus. After incubation the supernatant was discarded, and the cells were fixed with 3.5% formaldehyde (Sigma-Aldrich) in PBS for 30 min at room temperature, washed four times with PBS, and exposed to Hoechst 33342 at 10 μ M for 30 min at room temperature. Cell preparations were examined under UV illumination with a fluorescence microscope (Olympus Optical Co., Tokyo, Japan).

2.5. Measurement of reactive oxygen species

Cells were incubated with 10 μ M of DCFH diacetate (DCFH-DA) for 30 min, harvested by trypsinization, collected by centrifugation, and resuspended in PBS containing 2 μ g/mL propidium iodide (Sigma-Aldrich). After sorting out the viable cells, fluorescence intensity was measured by flow cytometry (Becton-Dickinson, San Jose, CA, USA) using excitation and emission wavelengths of 488 nm and 525 nm, respectively.

3. Results

3.1. Effect of ginsenoside-Rh2 on cell proliferation of cancer cells

Several recent reports have implicated the effect of ginsenoside-Rh2 on cancer cell death [24–26]. We examined the effect of

various ginsenosides, such as ginsenoside-Rc, Rd, Re, Rg3, Rh1, and Rh2, on cellular proliferation and growth using the MTT assay. HepG2 cells were exposed to increasing concentrations (25–50 μM) of each ginsenoside for 48 h. Among them, treatment of ginsenoside-Rh2 (25 μM or 50 μM) for 48 h induced a significant growth inhibition in HepG2 human hepatocellular carcinoma (Fig. 2A). Also, a more significant dose-dependent growth inhibitory effect is observed in cervical carcinoma (HeLa) than in any other cancer cell lines tested—hepatoma (HepG2), prostate carcinoma (DU145), and colon cancer (HCT116) cell lines—for 24 h treatment (Fig. 2B).

3.2. Ginsenoside-Rh2 enhances AMPK activity and AMPK plays an antiapoptotic role in ginsenoside-Rh2 apoptosis

As shown in Fig. 2, some cancer cells have differential sensitivity to ginsenoside-Rh2-induced apoptosis, raising questions regarding the specific mechanisms responsible for this sensitivity. Because several recent reports have implicated the role of AMPK in preventing apoptosis in various cancer cell type [21,22], we examined the ability of ginsenoside-Rh2 to enhance AMPK activity in a variety of cancer cells. To measure AMPK activity, we used phospho-specific (Phospho-Thr172) antibody for AMPK. As shown in Fig. 3, treatment with ginsenoside-Rh2 25 or 50 μM for 4 h significantly induces AMPK activation in HepG2, DU145, and HCT116 cells, but not in HeLa cells. Because HeLa cells do not induce AMPK activation and exhibit relatively more sensitivity to ginsenoside-Rh2-induced apoptosis (Fig. 2B), we examined the correlation with AMPK activity and cell death. The results show that pharmacological inhibition of AMPK, in the presence of the AMPK inhibitor (compound C), reduces cell viability in HepG2 cells. The combined treatment of compound C with ginsenoside-Rh2 (25 μM) resulted in lower cell viability than treatment with ginsenoside-Rh2 alone for the indicated periods. Apoptotic cells were assessed using MTT (Fig. 4A) and Hoechst 33342 staining (Fig. 4B). Additionally, it was shown through Western blot analysis that PARP cleavage was substantially increased in compound C-treated cells (Fig. 4C). Although ginsenoside-Rh2 treatment induces AMPK activation in HepG2 cells, it does not affect AMPK activity in HeLa cells, and thereby treatment with the AMPK inhibitor does not affect the degree of PARP cleavage (Fig. 4D). These results indicated that the AMPK signaling pathway is important in blocking ginsenoside-Rh2-induced apoptosis, and that AMPK plays a critical role as an antiapoptotic molecule.

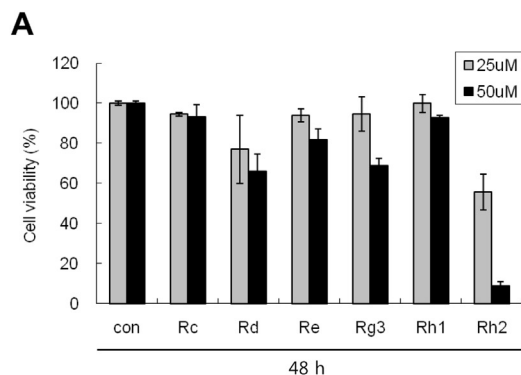


Fig. 2. Ginsenoside-Rh2 induces apoptosis in various cancer cells. (A) HepG2 cells were treated with 25 μM or 50 μM ginsenoside-Rc, Rd, Re, Rg3, Rh1, and Rh2 for 48 h. (B) HepG2 (a human hepatocellular carcinoma), DU145 (a human prostate carcinoma), HCT116 (a human colon carcinoma), and HeLa (a human cervix adenocarcinoma) cells were treated with the indicated concentration of ginsenoside-Rh2 for 24 h. Cell growth inhibition was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described in Materials and methods.

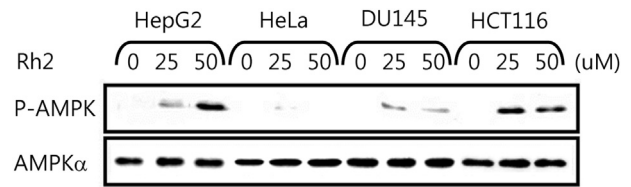


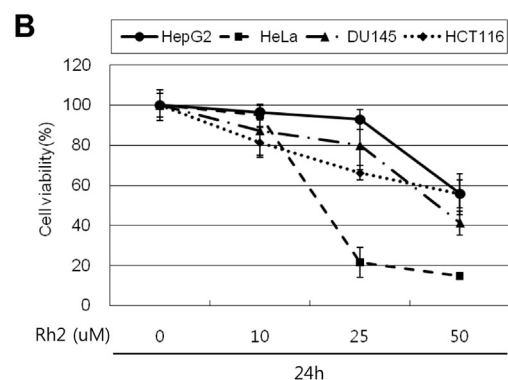
Fig. 3. Ginsenoside-Rh2 activates AMP-activated protein kinase (AMPK) in HepG2, DU145, HCT116 cells, but not HeLa cells. HepG2, HeLa, DU145, and HCT116 cells were treated with 25 μM or 50 μM ginsenoside-Rh2 for 4 h. Under these conditions, the phosphorylated and total form of AMPK was examined via Western blot assay using specific antibodies.

3.3. AMPK activation is mediated by ginsenoside-Rh2-induced reactive oxygen species generation

Recently, studies reported that AMPK is activated by reactive oxygen species (ROS) generation in various cell lines [27,28]. To investigate whether ginsenoside-Rh2 induces ROS production, and thereby affects AMPK activity, HepG2 cells were treated with 25 μM ginsenoside-Rh2 for 8 h, and ROS was then measured using flow cytometric analysis of DCFH-DA-stained cells. As shown in Fig. 5A, ginsenoside-Rh2 induces an increase in ROS level, and treatment of 10 μM NAC blocks ginsenoside-Rh2-induced ROS generation. We next examined the effect of ROS production on AMPK activity after treatment of 25 μM ginsenoside-Rh2 both with and without 10 μM NAC for 8 h. The result shows that NAC treatment completely blocks ginsenoside-Rh2-induced AMPK activation (Fig. 5B) in HepG2 cells. These results indicate that AMPK activation is mediated by ginsenoside-Rh2-induced ROS generation.

3.4. Ginsenoside-Rh2 activates ERK, JNK, and p38 MAPK

MAPKs are known to correlate with the pharmacological effects of ginsenosides. Ginsenoside-Rh2-induced late-phase activation of JNK is associated with the induction of apoptosis via the proteolytic dissociation of p21^{WAF/CIP1} from JNK1-containing complexes [29]. ERK activation inhibits ginseng metabolite, IH-901-induced apoptosis and cell cycle arrest, via COX-2 induction [30]. The anti-proliferative effect of ginsenoside-Rg1 is involved in the inhibition of ERK in cultured human arterial vascular smooth muscle cell [31]. Thus, we next examined whether the MAPK pathway is associated with ginsenoside-Rh2-induced apoptosis and the antiapoptotic effects of AMPK in HepG2 cells. As shown in Fig. 6A, ginsenoside-



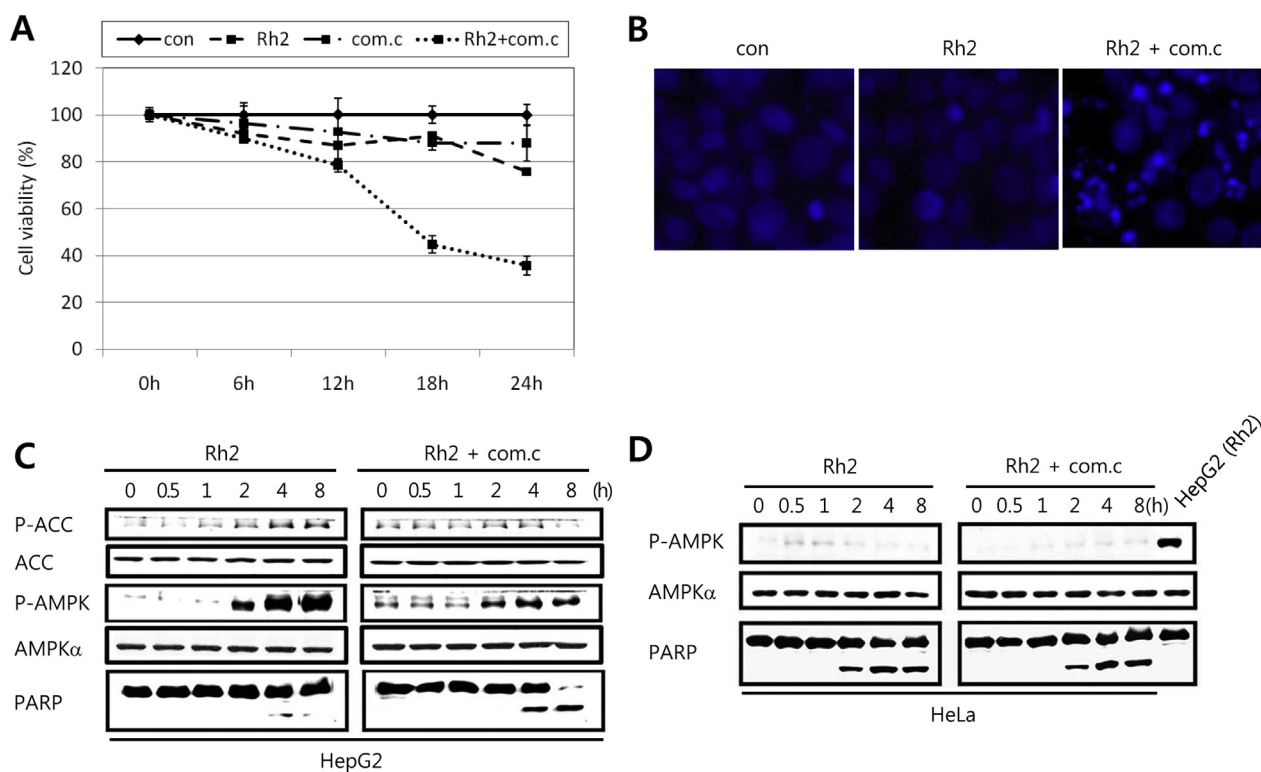


Fig. 4. Inhibition of AMP-activated protein kinase (AMPK) enhances ginsenoside-Rh2-induced apoptosis in HepG2 cells but not HeLa cells. (A) HepG2 cells were exposed to 25 μ M ginsenoside-Rh2 for the indicated period with or without 20 μ M compound C and then analyzed for apoptosis via MTT assay. (B) Cells were cotreated with 20 μ M compound C and 25 μ M ginsenoside-Rh2 for 8 h. The apoptotic cell death was examined with Hoechst staining by fluorescence microscope. HepG2 (C) and HeLa (D) cells were exposed to 25 μ M ginsenoside-Rh2 for the indicated period with or without 20 μ M compound C. Total cell extracts were prepared under the identical conditions and subjected to Western blot assay using antibody against the phosphorylated form of acetyl-CoA carboxylase (P-ACC), the phosphorylated form of AMPK (P-AMPK), AMPK α and PARP specific antibodies. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase.

Rh2 induces the activation of three MAPKs in a time-dependent manner. To determine whether the activity of the three MAPKs was involved in ginsenoside-Rh2-induced apoptosis, HepG2 cells were pretreated with 20 μ M PD98059, SB203580, and SP600152, a selective inhibitor of ERK, p38 MAPK, and JNK, respectively. Cotreatment with ginsenoside-Rh2 and SB203580 (p38 MAPK inhibitor) causes cell death to increase from 20% to 50%, compared with ginsenoside-Rh2 treatment alone, suggesting that the inhibition of p38 MAPK can enhance ginsenoside-Rh2-induced apoptosis in HepG2 cells. However, there was no observed effect of ERK or JNK inhibition on cell death.

To examine whether there was a correlation between AMPK and p38 MAPK activity, we investigated AMPK and p38 MAPK activation following each kinase inhibition by compound C or SB203580. As shown in Fig. 6C, inhibition of AMPK did not affect p38 MAPK activity, and inhibition of p38 MAPK did not affect AMPK activity, either. Therefore, it is likely that AMPK and p38 MAPK transmit its signal in an independent manner.

4. Discussion

Ginseng, the root of *P. ginseng*, is a medicinal herb that has been reported to have various biological effects, including anticarcinogenic activities. Ginseng extracts induce apoptosis, and decrease telomerase activity and cyclooxygenase-2 (COX-2) expression in human leukemia cells [32]. In addition, ginseng extracts suppress colon carcinogenesis induced by 1,2-dimethylhydrazine with inhibition of cell proliferation [33]. Among them, ginsenoside-Rh2 is recognized as a major active anticancer saponin [34]. Ginsenoside-Rg3 is known to metabolize to ginsenoside-Rh2 by human

intestinal bacteria [35]. In this regard, the anticancer activity of two compounds has been compared in many reports. In the case of Hep3B cells, these two compounds induce apoptosis through a mitochondrial pathway [36]. However, several reports demonstrated that ginsenoside-Rh2 showed a more potent anticancer activity than ginsenoside-Rg3 [37,38]. Ginsenoside-Rh2 treatment modulates the protein level of p21 and cyclin D, which results in a marked reduction in proliferation on MCF-7 human breast cancer cells [16]. Ginsenoside-Rh2 also induces apoptosis through the activation of p53 and the increase of the proapoptotic regulator, Bax, in colorectal cancer cells [37]. In addition, Ginsenoside-Rh2 markedly inhibits the viability of breast cancer cells (MCF-7 and MDA-MB-231) with G1 phase cell cycle arrest, which is caused by p15 Ink4B and p27 Kip1-dependent inhibition of cyclin-dependent kinases [10]. Although many studies describing the anticancer effect of ginsenoside-Rh2 have been conducted, much of its mechanism relating to anticancer activities remains unclear.

AMPK is a pleiotropic kinase that signals for both survival and apoptosis of cells. It plays a key role as a regulator of cellular energy homeostasis [39]. The kinase is activated in response to ATP depletions, such as those of glucose starvation, hypoxia, ischemia, and heat shock. Moreover, a proapoptotic function of AMPK was also reported, where the connection of AMPK with several tumor suppressors suggests that AMPK is a mediator of apoptosis. The LKB1 tumor suppressor that mutated in Peutz-Jeghers syndrome directly phosphorylates and activates AMPK [40,41]. The TSC2 tumor suppressor is directly phosphorylated by AMPK, and the AMPK-mediated phosphorylation of TSC2 has an important role in cell survival [42,43]. The present study focuses on identifying the mechanism that underlies the anticancer activity of ginsenoside-

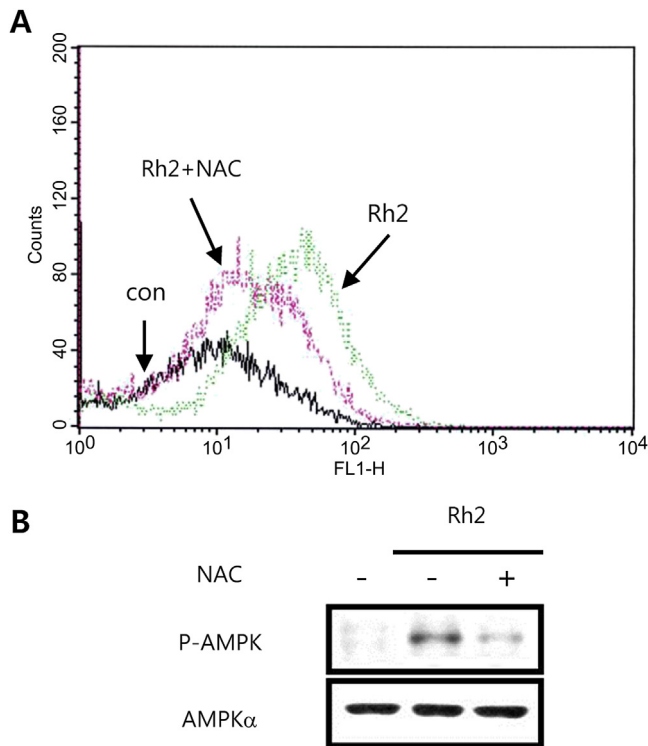


Fig. 5. AMP-activated protein kinase (AMPK) is activated by ginsenoside-Rh2-mediated reactive oxygen species (ROS) generation. (A) HepG2 cells were exposed to 25 μ M ginsenoside-Rh2 for 4 h, and then DCFH-DA (10 μ M) was added for 30 min. The changes in fluorescence intensity were measured by fluorescence-activated cell scanning analysis. (B) After exposure to 25 μ M ginsenoside-Rh2 for 4 h in the presence or absence of 1 mM NAC (*N*-acetyl-cysteine), the phosphorylated and total level of AMPK were examined. DCFH-DA, 2,7-Dichlorofluorescein-diacetate.

Rh2. In this study, we show that in HepG2 cells treated with ginsenoside-Rh2, AMPK activity is increased in a time- and dose-dependent manner (Figs. 3 and 4C). To confirm the role of AMPK in ginsenoside-Rh2-induced apoptosis, HepG2 cells were treated with ginsenoside-Rh2, and were then assessed for the degree of apoptosis according to the degree of variation in AMPK activity. In

this study, we have shown that AMPK activity is caused by ginsenoside-Rh2-mediated ROS generation (Fig. 5), and that it contributes to cancer cell growth and survival under treatment with ginsenoside-Rh2 (Fig. 4). These observations indicate that AMPK can function as an antiapoptotic molecule.

It is well documented that MAPK pathways modulate gene expression, mitosis, proliferation, metabolism, and apoptosis. Previous studies have demonstrated that MAPK signaling is involved in ginsenoside-mediated anticarcinogenesis. Ginsenoside Rg3 and Rh2 inhibit the proliferation of prostate cancer cells by modulating MAPK [17]. Ginsenoside Rb1 inhibits histamine release and IL-4 production induced by substance P, a neurotransmitter, via the ERK pathway [44]. A ginseng saponin metabolite, compound K, suppresses phorbol ester-induced matrix metalloproteinase-9 expression through the inhibition of MAPK signaling in human astrogloma cells [45]. Our results show that p38 MAPK activity is involved in ginsenoside-Rh2-mediated apoptosis (Fig. 6B,C). The induction of ginsenoside-Rh2-mediated apoptosis by p38 MAPK inhibitor SB203580 suggests that p38 MAPK signaling is important in protecting cancer cell against apoptosis. However, the molecular mechanism involved in the antiapoptotic role of p38 MAPK remains unclear and needs to be studied further.

Recently, several reports have also linked AMPK activity to p38 MAPK. AMPK activator AICAR increases glucose uptake by activating the p38 MAPK pathway, but the p38 MAPK inhibitor did not affect AMPK activation by AICAR in skeletal muscle [46]. The retinoic acid-mediated activation of p38 MAPK was inhibited by treatment with the AMPK inhibitor, compound C [47]. However, a further study suggests that AMPK activation leads to p38 MAPK inhibition. p38 MAPK is induced by the addition of cAMP to serum-starved H4IIE cells, and it is inhibited with AICAR treatment [48]. Even though several reports show that AMPK regulates p38 MAPK activity, the underlying mechanism of this interaction is not clearly understood. In this regard, we also examined if there is any cross-talk between AMPK and p38 MAPK (Fig. 6C), but there was no signaling crosstalk between these two kinases. Our present observations provide the rationale for a combination of AMPK and p38 MAPK inhibitors in the treatment of cancer, and future studies focusing on the molecular mechanism of AMPK and p38 MAPK in ginsenoside-Rh2-induced apoptosis would greatly extend our understanding of the chemotherapeutic potency of ginsenoside-Rh2 in human cancer.

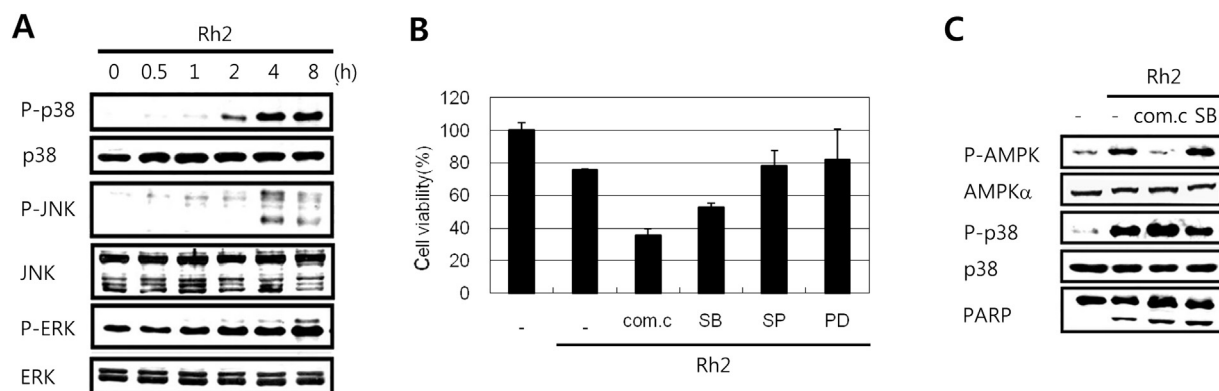


Fig. 6. Ginsenoside-Rh2 activates MAPKs, and both AMPK and p38 protect cells from ginsenoside-Rh2-induced apoptosis in HepG2 cells. (A) HepG2 cells were treated with 25 μ M ginsenoside-Rh2 for the indicated period and the phosphorylated and total form of the indicated protein including p38 MAPK, c-Jun NH₂-terminal kinase (JNK), ERK, was examined using specific antibodies. (B) Cells were pre-treated with each kinase inhibitor (20 μ M) compound C (AMPK), SB203580 (p38 MAPK), SP600125 (JNK), and PD98059 (ERK) for 30 min and then cells were exposed for 24 h to 25 μ M ginsenoside-Rh2 for 24 h. Cell viability was measured by MTT assay. (C) Cells were pretreated with each kinase inhibitor (20 μ M) compound C (AMPK) and SB203580 (p38 MAPK) for 30 min and were then exposed to 25 μ M ginsenoside-Rh2 for 4 h, and then total cell extracts were subjected to Western blot assay using phosphorylated and total form of the indicated protein and PARP specific antibodies. AMPK, AMP-activated protein kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

This work was supported by a grant from the Kyung Hee University in 2010 (KHU-20100849).

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