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Ginseng saponin metabolite induces apoptosis in MCF-7 breast cancer cells through the modulation of AMP-activated protein kinase

Areum Daseul Kim[a], Kyoung Ah Kang[b], Rui Zhang[b], Chae Moon Lim[b], Hee Sun Kim[c], Dong Hyun Kim[d], You Jin Jeon[a], Chang Hyun Lee[b], Jinny Park[e], Weon Young Chang[b,*], Jin Won Hyun[b,**]

[a] Department of Marine Life Science, Jeju National University, Jeju-si 690-756, Republic of Korea
[b] Jeju National University School of Medicine, Jeju-si 690-756, Republic of Korea
[c] Department of Neuroscience, College of Medicine, Ewha Womans University, Seoul 110-783, Republic of Korea
[d] Department of Microbial Chemistry, College of Pharmacy, Kyung Hee University, Seoul 130-701, Republic of Korea
[e] Division of Hematology and Oncology, Department of Internal Medicine, Gachon University of Medicine Science, Gil Hospital, Incheon 405-760, Republic of Korea

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ABSTRACT

Previous studies have shown that the ginseng saponin metabolite, Compound K (20-*O*-D-glucopyranosyl-20(S)-protopanaxadiol, IH901), suppresses proliferation of various cancers and induces apoptosis. AMP-activated protein kinase (AMPK) is a sensor of cellular energy states and is involved in apoptosis of cancer cells. We hypothesized that Compound K may exert cytotoxicity in MCF-7 human breast cancer cells through modulation of AMPK, followed by a decrease in cyclooxygenase-2 (COX-2) expression. Compound K inhibited cell growth, induced apoptosis via generation of reactive oxygen species (ROS), as well as decreasing COX-2 expression and prostaglandin E_2 (PGE_2) levels. These effects of Compound K were induced via an AMPK-dependent pathway and were abrogated by a specific AMPK inhibitor. These results suggest that Compound K induced apoptosis by modulating AMPK–COX-2 signaling in MCF-7 human breast cancer cells.

1. Introduction

Ginsenosides have been shown to inhibit tumour cell proliferation and tumour growth, to induce differentiation and apoptosis, and to inhibit tumour cell invasion and metastasis (Iishi et al., 1997; Kikuchi et al., 1991; Lee et al., 1996; Park et al., 1997; Shinkai et al., 1996). Intestinal bacteria have been shown to form ginsenoside metabolites after oral administration of ginseng extract in both humans and rats (Hasegawa et al., 1996). One of the major metabolites detected in the urine and blood after administration of total ginsenoside in rats is [20-*O*-(β-D-glucopyranosyl)-20(S)-protopanaxadiol] (Compound K, IH-901; Fig. 1). Recently, it has been reported that Compound K was absorbed into blood 24 h after oral administration of 12 g of ginseng powder, with average values for maximum plasma concentration (27.89 ± 24.46 ng/ml), maximum drug concentration time (10.76 ± 2.07 h), and area under the plasma drug concentration (221.98 ± 221.42 μg h/ml) (Lee et al., 2009). Compound K is active in biological systems and is also known to inhibit glucose uptake in tumour cells, to possess chemopreventive activity against chemical carcinogens, to inhibit metastasis *in vivo*, and to inhibit tumour growth through inhibition of TPA-induced cyclooxygenase-2 expression (Hasegawa et al., 1994, 1997; Lee et al., 1998; Lee et al., 2005; Yim et al., 2005). We recently reported that Compound K exhibits cytotoxicity by the induction of apoptosis and cell cycle arrest at the G_1 phase, by a caspase-dependent pathway via mitochondria disruption, and by inhibition of telomerase activity and the combined treatment of Compound K and radiation enhances human lung cancer cell death (Kang et al., 2005a,b,c; Chae et al., 2009). Cyclooxygenase-2 (COX-2), the central enzyme in prostanoid biosynthesis, is involved in the first step of prostanoid synthesis from arachidonic acid (Attar and Lin, 1987). It catalyzes the conversion of arachidonic acid to prostaglandin H_2, which is further converted to several other prostaglandins and plays an essential role in carcinogenesis and inflammation (Cho et al., 2005; Lee et al., 2007). COX-2 is over-expressed in many cancers including breast cancer and colon cancer. Selective COX-2 inhibitors effectively prevent proliferation and angiogenesis and induce apoptosis in human breast cancer and colon cancer cells (Mazhar et al., 2006; Das et al., 2007). AMP-activated protein kinase (AMPK) belongs to a family of serine/threonine protein kinases. AMPK plays an essential role as an energy-sensor mainly in ATP-deprived conditions (Hardie et al., 1998). Therefore, AMPK plays a major protective role under metabolic stressed conditions such as hypoxia, ischemia, and an up-regulated state of reactive

* Corresponding author: Tel.: +82 064 717 1487; fax: +82 064 717 1511.
** Corresponding author: Tel: +82 064 754 3838; fax: +82 064 726 4152.
E-mail addresses: orkorea@jejunu.ac.kr (W.Y. Chang), jinwonh@jejunu.ac.kr (J.W. Hyun).

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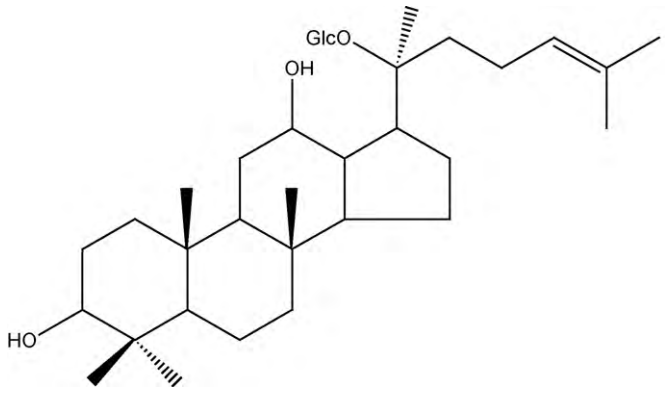

Fig. 1. Chemical structure of Compound K [20-*O*-D-glucopyranosyl-20(S)-protopanaxadiol].

oxygen species (ROS) (Park et al., 2009; Lee et al., 2003; Hwang et al., 2004). In the activated state, AMPK down-regulates several anabolic enzymes and thus shuts down the ATP-consuming metabolic pathways (Kemp et al., 2003). AMPK activation regulates apoptosis in multiple cancer cells by a signaling pathway that includes up-regulation of p53 and p21 proteins, activation of caspases, inhibition of COX-2 related to growth, and proliferation of cancer cells (Hwang et al., 2005, 2006). In this study, we examined the role of Compound K inhibition of cell growth, induction of apoptosis via generation of ROS, and the possible involvement of an AMPK–COX-2 signaling pathway exerting these regulatory processes. Based on the findings of this study, we propose that the anticancer activity of Compound K involves AMPK expression that targets COX-2 regulation in breast cancer cells.

2. Materials and methods

2.1. Preparation of Compound K

Compound K was prepared by the incubation of protopanaxadiol type ginsenosides with Bacteroides JY-6, a human intestinal bacterium, subcultured in a general anaerobic medium for 24 h at 37 °C. The incubated medium was extracted with *n*-butanol. The supernatant was concentrated in vacuo and processed using silica gel column chromatography with chloroform/methanol/H_2O (65:35:10). The isolated Compound K was characterized by mass spectroscopy and ^1H and ^{13}C nuclear magnetic resonance (NMR) spectrometry.

2.2. Reagents

2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA), propidium iodide (PI), *N*-acetylcysteine (NAC), Hoechst 33342, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT), primary rabbit phospho-acetyl-CoA-carboxylase (ACC) (Ser79), phospho-AMPKα (Thr172), COX-2, and β-actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine chloride (JC-1) was purchased from Molecular Probes (Leiden, The Netherlands). Prostaglandin E_2 (PGE_2) enzyme assay kit was purchased from R&D Systems (Minneapolis, MN, USA), and Compound C was from Calbiochem Co. (San Diego, CA, USA).

2.3. Cell culture

Human breast cancer cells (MCF-7) and human breast normal cells (MCF-10A) were obtained from the American Type Culture Collection (Rockville, MD, USA), and the cells were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2 in air, and cultured in RPMI 1640 containing 0.1 mM non-essential amino acids, 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml).

2.4. Cell viability

The effect of Compound K on cell viability was determined using the MTT assay. Cells were treated with Compound K. Forty-eight hours later, 50 μl of MTT stock solution (2 mg/ml) was added and after incubation for 4 h the absorbance at 540 nm was then measured on a scanning multi-well spectrophotometer (Carmichael et al., 1987).

2.5. Flow cytometric analysis

The amount of apoptotic sub-G_1 hypodiploid cells was determined by flow cytometry (Nicoletti et al., 1991). Cells were treated with Compound K for 24 h, and harvested cells were washed twice with phosphate buffered saline (PBS) and fixed in 70% ethanol for 30 min at 4 °C. The cells were incubated in 50 mg/ml propidium iodide solution and 50 μg/ml RNase A in the dark for 30 min at 37 °C. A flow cytometric analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). The sub-G_1 hypodiploid cells were assessed based on the histograms generated by the Cell Quest and Mod-Fit computer programs.

2.6. Nuclear staining with Hoechst 33342

One and a half microliters of Hoechst 33342 (stock 10 mg/ml), which is a DNA-specific fluorescent dye, was added to each well, and the cells were incubated for 10 min at 37 °C. The stained cells were visualized under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera.

2.7. Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed using an *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer (Zheng et al., 2006). Briefly, image analysis for the TUNEL assay was achieved by seeding cells on chamber slides at 2×10^5 cells/well. Sixteen hours after plating, cells were treated with Compound K. Twenty-four hours later, chamber slides were fixed with 4% paraformaldehyde for 1 h at 15–25 °C and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. After washing in PBS, sections were incubated with the TUNEL reaction mixture for 1 h at 37 °C. After washing with PBS, the stained cells were mounted onto a microscope slide in mounting medium (DAKO, Carpinteria, CA, USA). The stained cells were then observed under a fluorescent microscope, which was a fluorescent microscope (Olympus IX 70, Olympus Optical Co., Japan).

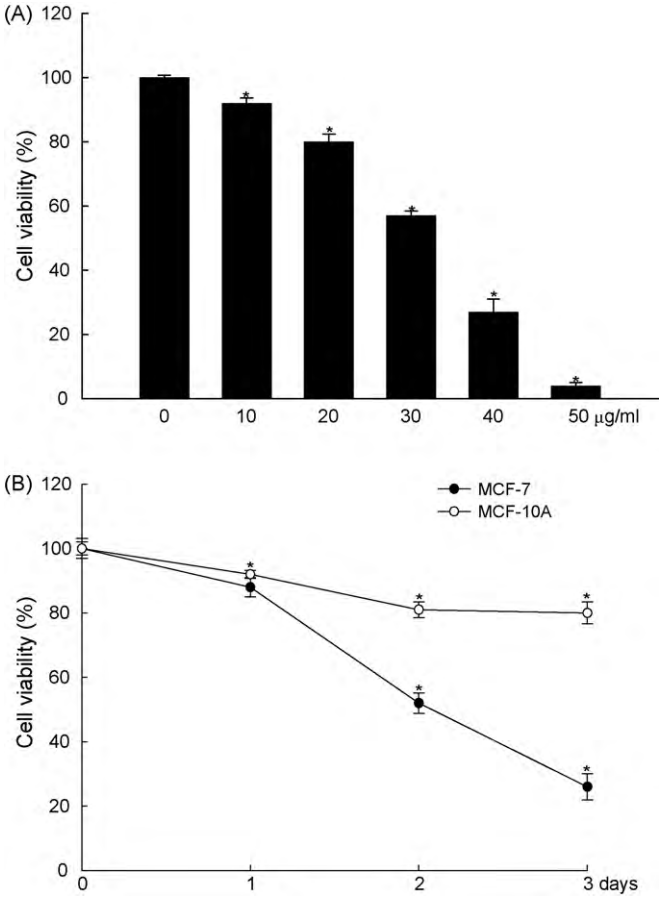

Fig. 2. Cytotoxic effect of Compound K against human breast cells. Cell viability (A) at the indicated concentrations of Compound K at 48 h and (B) at the indicated times with Compound K at 35 μg/ml was assessed using MTT test. *Significantly different from control ($p < 0.05$)

2.8. DNA fragmentation

Cellular DNA fragmentation was assessed by analysis of the cytoplasmic histone-associated DNA fragmentation kit from Roche Diagnostics according to the manufacturer's instructions.

2.9. Mitochondrial membrane potential ($\Delta\psi_m$) analysis

Cells were stained with JC-1 (10 μg/ml), and the stained cells were analyzed by flow cytometry (Troiano et al., 2007). In addition, for image analysis, the JC-1-stained cells were mounted with mounting medium (DAKO, Carpinteria, CA, USA). Microscopic images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) on a confocal microscope.

2.10. Intracellular reactive oxygen species (ROS) measurement

The DCF-DA method was used to detect the levels of intracellular ROS (Rosenkranz et al., 1992). The image analysis for the generation of intracellular ROS was achieved by seeding the cells on a cover-slip loaded six well plate at 1×10^5 cells/well. 24 h after plating, the cells were treated with 1 mM NAC, and after 1 h, Compound K added. After changing the media, 100 μM DCF-DA was added to each well and incubated for an additional 30 min at 37 °C. After washing with PBS, the stained cells were mounted onto microscope slides in mounting medium (DAKO, Carpinteria, CA, USA). Microscopic images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) on a confocal microscope. In addition, after the addition of 25 μM of DCF-DA solution for 45 min, the fluorescence of 2′,7′-dichlorofluorescein was detected by flow cytometry.

2.11. Western blot analysis

The lysates were electrophoresed, and blots were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibody and then with secondary immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.12. PGE_2 assay

PGE_2 amount was measured with the PGE_2 enzyme assay kit from R&D Systems according to the manufacturer's instructions.

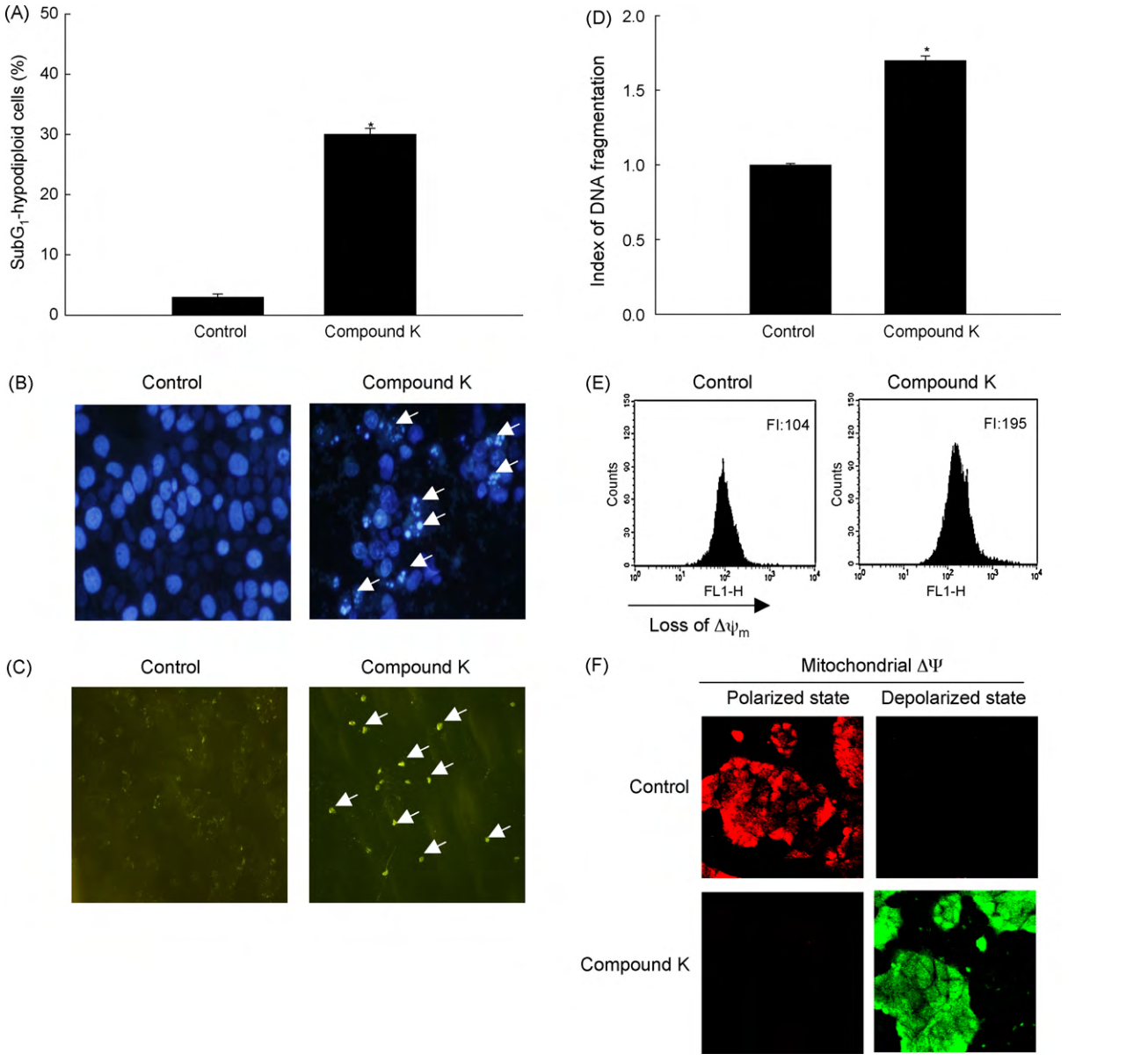

Fig. 3. Induction of apoptosis by Compound K treatment. (A) The apoptotic sub-G_1 DNA content was detected by flow cytometry after propidium iodide staining. (B) Apoptotic body formation was observed under a fluorescence microscope after Hoechst 33342 staining and apoptotic bodies are indicated by arrows. (C) Apoptotic cells were detected by TUNEL staining assay. The arrows point to TUNEL-positive cells. (D) DNA fragmentation was quantified with an ELISA kit. (E) $\Delta\psi_m$ was analyzed by flow cytometry and (F) confocal microscopy after staining cells with JC-1 dye.

2.13. Statistical analysis

All measurements were made in triplicate and all values were expressed as the mean ± standard error of the mean (SEM). The results were subjected to an analysis of variance (ANOVA) using the Tukey test to analyze the difference. $p < 0.05$ was considered significant.

3. Result

3.1. Compound K inhibits cell growth of MCF-7 breast cancer cells

Compound K inhibited MCF-7 cell growth in a dose-dependent manner at 10, 20, 30, 40, and 50 μg/ml at 48 h, and the concentration at which 50% growth inhibition (IC_{50}) was 35 μg/ml (Fig. 2A). At 35 μg/ml, Compound K also inhibited MCF-7 cell growth in a time-dependent manner, but did not show cytotoxicity in MCF-10A normal breast cells compared to MCF-7 cells (Fig. 2B).

3.2. Compound K induces apoptosis

We investigated whether the cytotoxicity of Compound K was associated with induction of apoptosis. Sub-G_1 hypodiploid cells, apoptotic bodies, TUNEL-positive cells, and DNA fragmentation, which are indicators of apoptosis phenomenon, were observed in Compound K-treated cells (Fig. 3A–D). The apoptotic pathway requires an alteration of $\Delta\psi_m$ leading to mitochondrial membrane permeabilization and followed by a release of cytochrome *c* and activation caspases (Rosenkranz et al., 1992; Chipuk et al., 2006; Green and Reed, 1998). Therefore we measured changes in $\Delta\psi_m$ levels. The flow cytometric data showed that Compound K treatment resulted in the loss of $\Delta\psi_m$ compared to control, as substantiated by increased JC-1 dye fluorescence (Fig. 3E). These data were consistent with the confocal microscopy data (Fig. 3F). JC-1-stained control cells as red fluorescence (polarized state of $\Delta\psi_m$) in the mitochondria; however, Compound K-treated cells were stained as green fluorescence (depolarized state of $\Delta\psi_m$), suggesting that Compound K changes the $\Delta\psi_m$ levels.

3.3. Compound K induces intracellular ROS

ROS can change the mitochondrial permeability transition and result in $\Delta\psi_m$ loss, leading to apoptosis (Crompton et al., 2002; Cadenas and Davies, 2000). The red fluorescence intensity of ROS was enhanced in Compound K-treated cells compared to control; however, NAC treatment scavenged Compound K-generated ROS (Fig. 4A). This pattern was also confirmed by flow cytometry, showing that Compound K-treated cells that were pretreated with NAC

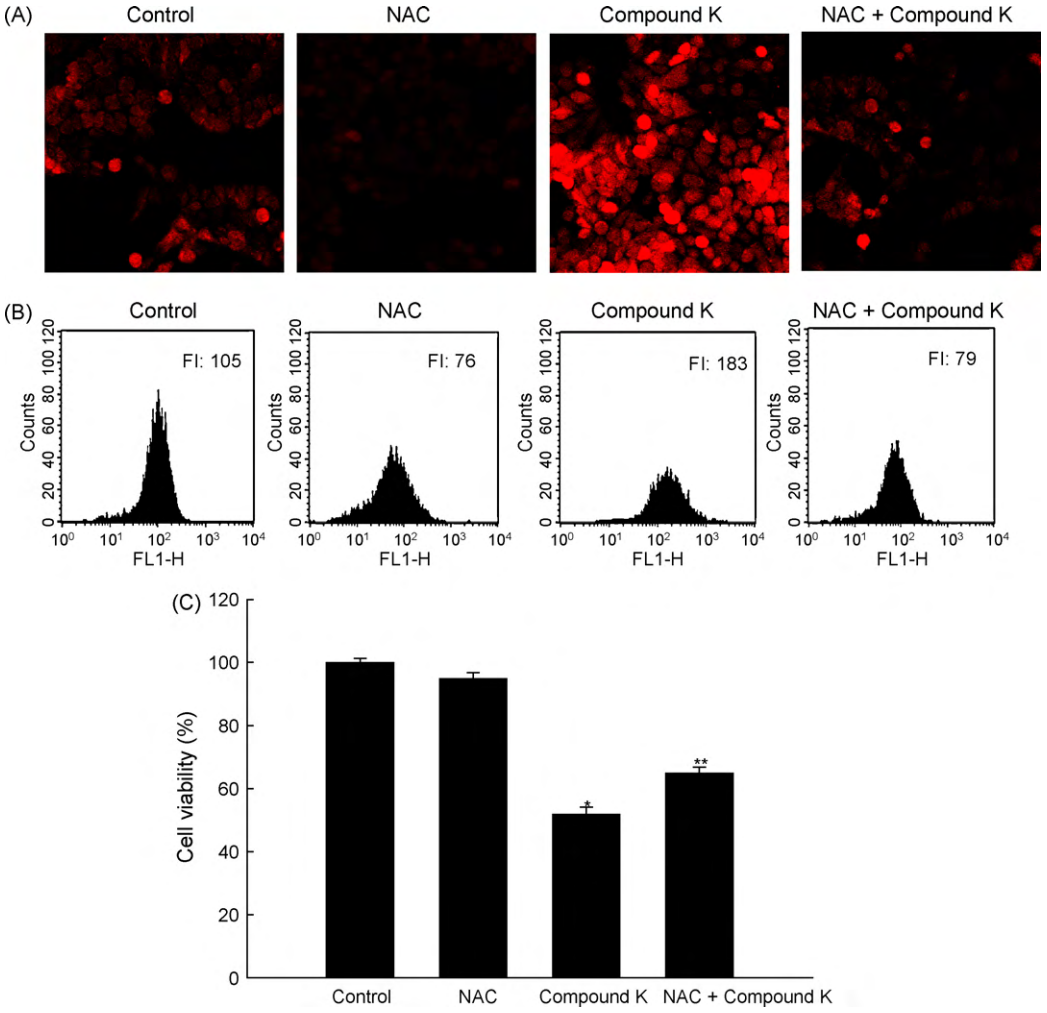

Fig. 4. Intracellular ROS generation by Compound K treatment. (A) The intracellular ROS generated were detected by confocal microscopy after DCF-DA treatment. The representative confocal images illustrate the increase in red fluorescence intensity of DCF produced by ROS in Compound K-treated cells compared to the control and the reduced fluorescence intensity in Compound K-treated cells with NAC (original magnification, 400×). (B) The intracellular ROS generated were detected by a flow cytometry after DCF-DA treatment. FI indicates the fluorescence intensity of DCF-DA. (C) After treatment with NAC or/and Compound K, the cell viability was assessed by MTT assay. *Significantly different from control ($p < 0.05$) and **significantly different from Compound K-treated cells ($p < 0.05$).

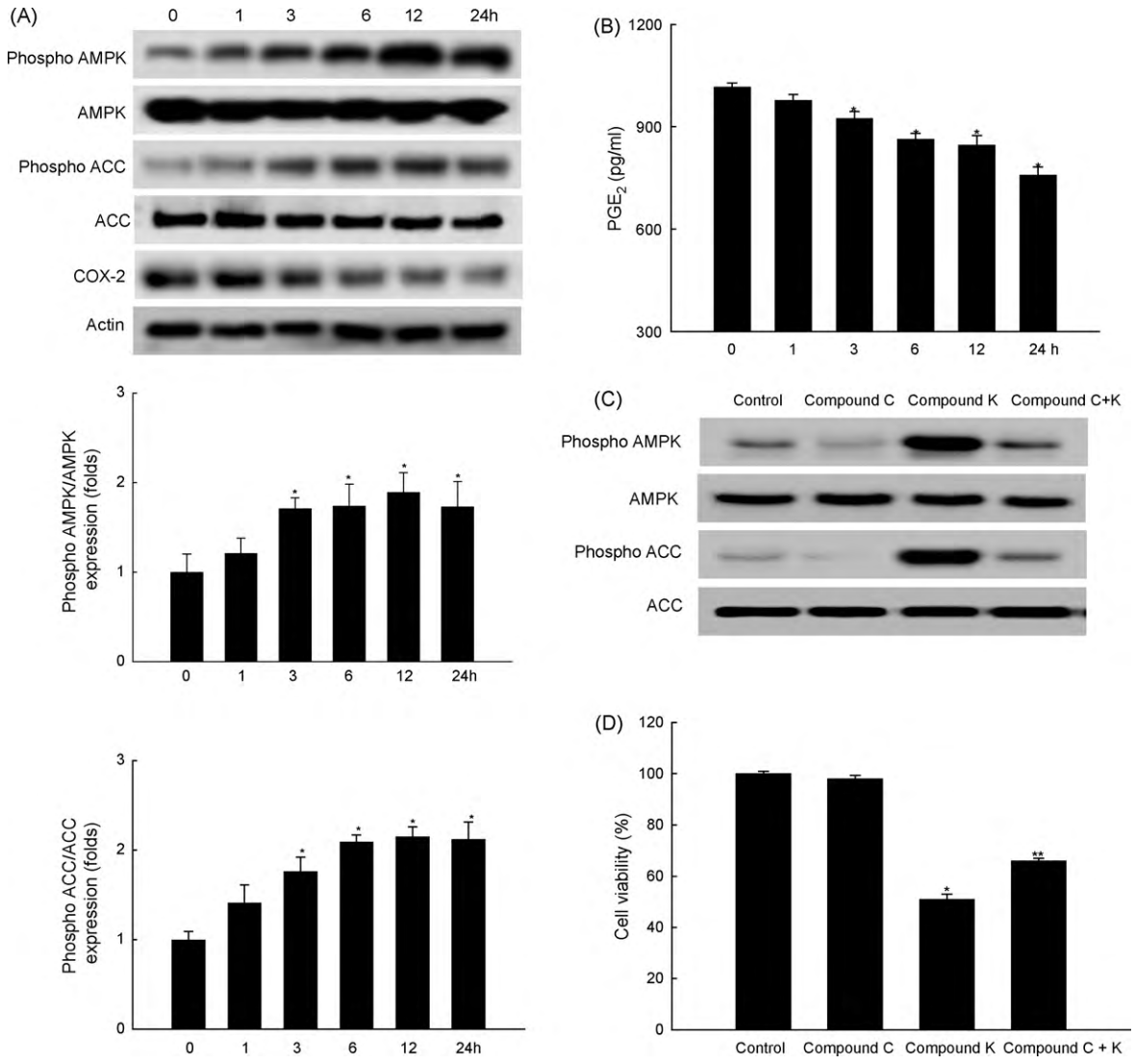

Fig. 5. Activation of AMPK by Compound K treatment. (A) The AMPK activity is determined by the expression level of phospho-AMPK, phospho-ACC, and COX-2 in Western blot assays. (B) The amount of PGE_2 was measured with an ELISA kit. (C) After treatment with Compound C or/and Compound K, the expression levels of phospho-AMPK and phospho-ACC were assessed by Western blot assays. (D) After treatment with Compound C or/and Compound K, the cell viability was assessed by MTT assay. *Significantly different from control ($p < 0.05$) and **significantly different from Compound K-treated cells ($p < 0.05$).

exhibited an ROS fluorescence intensity of 79 values, compared to 183 values in Compound K-treated cells (Fig. 4B). Furthermore, cell survival was increased to 64% in NAC-pretreated, Compound K-treated cells, compared to 52% in Compound K-treated cells (Fig. 4C). This result suggests that ROS generated by Compound K induce the cell death.

3.4. Compound K activates AMPK signaling pathway

Compound K significantly induced the activation of AMPK (phospho-AMPK) in a time-dependent manner, and this active form was confirmed by the level of phosphorylation of ACC, which is a phosphorylation site of AMPK (Fig. 5A). Compound K significantly inhibited the level of COX-2 expression in a time-dependent manner and abrogated basal PGE_2 production in a time-dependent manner (Fig. 5A and B). Compound C, a specific AMPK inhibitor, significantly attenuated both phospho forms of AMPK and ACC induced by Compound K treatment (Fig. 5C). Furthermore, Compound C significantly increased 65% of cell viability inhibited by Compound K treatment (52% of cell viability) (Fig. 5D). These results indicate that AMPK may play a role in Compound K-induced cytotoxicity against MCF-7 cells.

4. Discussion

Ginsenosides have been used as a traditional herbal medicine in Asian countries and they are now more widely used for preventive and therapeutic purposes. Recently, ginsenoside metabolites, formed by intestinal bacteria after the oral administration of ginseng extract in humans and rats (Hasegawa et al., 1996; Bae et al., 2000, 2002), were found to have many biological activities, including anti-inflammatory, anti-allergic, anti-diabetic, and anti-cancer activities in cells or animals (Lee et al., 2000; Choi et al., 2002). Compound K is an active metabolite of ginsenosides and exhibits anti-tumour effects against various cancer cells (Hasegawa et al., 1994, 1997; Lee et al., 1998, 2005; Yim et al., 2005; Kang et al., 2005a,b,c; Chae et al., 2009; Choi and Choi, 2009; Kim et al., 2008; Choo et al., 2008). Apoptosis is an important biological mechanism that contributes to the maintenance of the integrity of multi-cellular organisms and is dependent on the expression of

cell-intrinsic suicide machinery. Mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During this process, the phosphatidylserine in inner membrane was externalized and the electrochemical gradient across the mitochondrial membrane collapsed (Lugli et al., 2005). The collapse is thought to occur through the formation of pores in the mitochondria by dimerization of Bax or activation of Bid, Bak, or Bad proteins. Activation of these pro-apoptotic proteins is accompanied by the release of cytochrome *c* into the cytosol (Narita et al., 1998; Luo et al., 1998). JC-1 is a unique cationic dye to signal the loss of $\Delta\psi_m$. In healthy cells, JC-1 stains the mitochondria bright red. The negative charge established by the intact $\Delta\psi_m$ allows the lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates. When the critical concentration is exceeded, J-aggregates form, which become fluorescent red. However, in apoptotic cells, the $\Delta\psi_m$ collapses, and the JC-1 cannot accumulate within the mitochondria. In these cells, JC-1 remains in the cytosol as a green fluorescent monomeric form. Compound K-treated MCF-7 cells exhibited significant loss of $\Delta\psi_m$ as shown by confocal microscopic data and also showed other apoptotic characteristics: sub-G_1 hypodiploid cells, apoptotic body formation, TUNEL-positive cells, and DNA fragmentation. Several anticancer agents for cancer treatment have been shown to cause increased cellular ROS generation, and increased intracellular ROS cause loss of mitochondria membrane permeability, resulting in induction of apoptosis (Kang et al., 2004; Pelicano et al., 2004). In our system, Compound K generated significant ROS, and these generated species were abolished by NAC treatment. ROS are upstream of the AMPK activation signal and are implicated in the inhibition of COX-2 (Choi et al., 2001). Intracellular ROS can also be generated by numerous external stimuli and AMPK is highly sensitive to oxidative stress because increased cellular ROS change the AMP level, which lead to rapid AMPK activation. The involvement of AMPK in the inhibition of tumorigenesis can modulate the regulation of COX-2 (Yun et al., 2005). Compound K activated AMPK and thus abrogated the expression of COX-2 and PGE_2 production, and AMPK inhibitor (Compound C) protected MCF-7 cells against the cytotoxic effect of Compound K via attenuation of phospho-AMPK expression induced by Compound K, suggesting that AMPK is an important regulator in Compound K-induced cell death.

The present study demonstrates that Compound K exhibits cytotoxic effects via AMPK activation, ROS generation, and induction of apoptosis.

Conflicts of interest

The authors state that there are no conflicts of interest.

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