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Compound K Induces Apoptosis via CAMK-IV/AMPK Pathways in HT-29 Colon Cancer Cells

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Although compound K (CK), an intestinal metabolite of ginseng protopanaxadiol saponins, has been known to induce apoptosis in various cancer cells, association of AMP-activated protein kinase (AMPK) with apoptosis in HT-29 colon cancer cells remains unclear. We hypothesized that CK may exert an anticancer activity through modulating the AMPK pathway in HT-29 cells. CK-induced apoptosis was associated with the disruption of the mitochondrial membrane potential, release of apoptogenic factors (cytochrome *c* and apoptosis-inducing factor) from mitochondria, and cleavage of caspase-9, caspase-3, caspase-8, Bid, and PARP proteins. This apoptotic effect of CK on colon cancer cells was found to be initiated by AMPK activation, and AMPK was activated through phosphorylation by Ca^{2+}/calmodulin-activated protein kinase-IV (CAMK-IV). Treatment of HT-29 cells with compound C (AMPK inhibitor) or siRNA for AMPK completely abolished the CK-induced apoptosis. STO-609, CAMKs inhibitor, also attenuated CK-induced AMPK activation and apoptosis. In conclusion, the present study demonstrates that CK-mediated cell death of HT-29 colon cancer cells is regulated by CAMK-IV/AMPK pathways, and these findings provide a molecular basis for the anticancer effect of CK.

KEYWORDS: Compound K; AMPK; CAMK-IV; apoptosis; HT-29 colon cancer cells

INTRODUCTION

Cancer is a growing health problem around the world, and colon cancer is one of the leading causes of cancer-related deaths in developed countries (*1*). Cancer chemoprevention by medicinal plants has been investigated by many groups, and *Panax ginseng* has been implicated in the prevention of human carcinogenesis possibly through its inhibitory activities on cell proliferation or survival. Compound K (CK) is an active metabolite of protopanaxadiol-type saponins in the intestine after oral administration and is the major form of protopanaxadiol saponins absorbed in the body (**Figure 1**). CK has been shown to enhance the efficacy of anticancer drugs in cancer cells previously resistant to several anticancer drugs (*2*), to exhibit antigenotoxic and anticlastogenic activity in rats concurrently treated with benzo(*a*)pyrene (*3*), and to induce apoptosis (*4*−*6*). These studies reported that the anticancer activity of CK is ascribed to the induction of apoptosis. However, the detailed mechanism of CK-induced apoptosis is still poorly understood.

AMP-activated protein kinase (AMPK) belongs to a family of serine/threonine kinases and is a fuel sensor monitoring the AMP/ATP ratio to maintain cellular homeostasis (*7*). Several metabolic stresses, including hypoxia, exercise, and starvation, lead to activation of the AMPK pathway (*8*, *9*). Recently, several groups have documented the strong pro-apoptotic potential of AMPK in conditions such as AICAR (AMPK activator)-treated cells or constitutively active AMPK mutants (*10*). These results suggest that AMPK signaling could be a potential therapeutic target for cancer.

In the present study, we asked whether AMPK is associated with the CK-induced apoptosis. We have found the effects of CK on AMPK activation and inducing apoptosis, and CK-induced apoptosis was blunted in the presence of either AMPK inhibitor (compound C) or small interfering RNA for AMPK. In addition, CK-induced phosphorylation of AMPK was prevented by STO-609, CAMKs inhibitor. These findings indicate that CAMK-IV/AMPK pathways associate with apoptosis induced by CK in HT-29 cells and explore potential targets for human colon cancer.

MATERIALS AND METHODS

Chemicals. CK was obtained from the Central Research Center, ILHWA Pharmaceutical Co. (Guri, Korea), and dissolved in 0.1% dimethylsulfoxide (DMSO). Antibodies against CAMK-IV, phospho-Ser/Thr, AMPK, phospho-AMPK, acetyl-CoA carboxylase (ACC), phospho-ACC, and cleavaged caspase-3, caspase-8, and caspase-9 were purchased from Cell Signaling Technology (Beverly, MA); anti-actin, Bid, Bcl-2, PARP, AMPKα, siRNA, control siRNA, RNase, and protein A/G Sepharose bead were from Santa Cruz Biotechnology (Santa Cruz, CA); and the siRNA transfection reagent was from Genlantis, Inc. (San Diego, CA). Compound C and STO-609 were bought from Calbiochem (Darmstadt, Germany). Protein extraction and ECL kits were supplied

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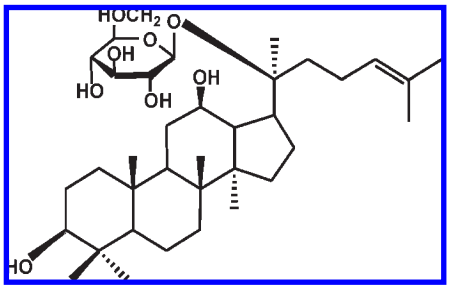

Figure 1. Chemical structure of CK.

from Intron Biotechnology, Inc. (Beverly, MA). Other reagents and chemicals were of analytical grade.

Cell Culture and Preparation of Whole Cell Lysates. The human colon cancer HT-29 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). HT-29 cells were cultured in RPMI (GibcoBL, Grand Island, NY) containing 10% fetal bovine serum, 100 unit/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 95% air and 5% CO_2 at 37 °C. For preparation of whole cell lysates to detect phosphoproteins, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed using a protein extraction kit. Insoluble protein was removed by centrifugation at 10000*g* for 20 min, and the protein concentration in cell lysates was measured using a Bio-Rad protein assay kit (Hercules, CA).

Cell Cycle Analysis. After treatment, both floating and attached cells were collected by low-speed centrifugation, washed in PBS, and then fixed in 70% ethanol at 4 °C overnight. Ethanol-fixed cells were resuspended in PBS containing 0.1 mg/mL RNase and incubated at 37 °C for 30 min. The pelleted cells were subsequently stained with 50 μg/mL propidium iodide (PI) for 30 min at room temperature. The relative DNA content per cell was analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Immunoprecipitation. A total of 300 μg of cell lysate was precleared with protein A/G-Sepharose beads, and supernatant was incubated with CAMK-IV antibody for 4 h at 4 °C. The reaction mixture was then mixed with protein-A/G Sepharose beads, incubated for 2 h at 4 °C, and centrifuged at 1000*g* for 1 min, and the precipitate was collected. The precipitate was washed twice with PBS; 1× sodium dodecyl sulfate (SDS) buffer was added and boiled for 1 min; and then the supernatant was collected. The phosphorylation of CAMK-IV was detected by Western blot.

Subcellular Fractionation. The method for the preparations of nucleus and cytosol fraction was modified from a previous report (*11*). HT-29 cells were washed with ice-cold PBS and resuspended in ice-cold lysis buffer [20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH at pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 10 μg/mL of pepstatin A and leupeptin] containing 250 mM sucrose for 30 min on ice. Cells were sonicated 3 times during this period. After centrifugation 10 min at 3500*g*, the supernatant (cytosol) was collected and stored at −70 °C for analysis. The pellet fraction (nucleus) was solubilized with a protein extraction kit and centrifuged at 10000*g* for 20 min at 4 °C. The supernatant was collected and stored at −70 °C for analysis.

Western Blot Analysis. Equal amounts of protein (40 μg/lane) were resolved by 10% SDS−polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and probed with appropriate primary and horseradish-conjugated secondary antibodies, as described previously (*12*). Anti-actin antibody was used as a loading control. The immunoreactive bands were visualized by enhanced chemiluminescence and quantified by densitometric analysis.

RNA Interference. To knock down the endogenous AMPKα, HT29 cells were transiently transfected with 10 nM of chemically synthesized siRNA targeting AMPKα1/2 or with non-silencing control siRNA according to the recommendations of the manufacturer. Transfected cells were used for Western blot analysis.

Statistical Analysis. Results are representative of at least three independent experiments performed in triplicate and are expressed as mean ± standard error (SE). Comparisons between groups were analyzed using Student's *t* test and considered significantly different when $p < 0.05$.

RESULTS

CK Induces Apoptosis in HT-29 Cells. To evaluate the effect of CK on the proliferation of human colon cancer cells, we examined the apoptotic characteristics of the CK-treated human colon cancer cell population using flow cytometry to quantitatively analyze their cell cycle phase distribution. HT-29 cells were treated with CK at 10, 25, and 50 μM for 24 h and compared to the untreated control. As shown in **Figure 2A**, the apoptotic sub-G_1 fraction was increased from 10.33 to 36.48% in HT-29 cells, whereas only 3.25% of the cells were in the sub-G_1 phase of the control group. These results indicate that CK induces apoptosis in HT-29 cells.

CK Induces Apoptosis via the Mitochondria-Dependent Pathway. In chemical-induced apoptosis, mitochondria play a central role in the commitment of cells to apoptosis through cytochrome-*c*-dependent or -independent pathways. To elucidate the molecular mechanism of CK-induced apoptosis in HT-29 cells, we examined the expression of proteins associated with apoptosis. HT-29 cells were treated with 50 μM CK for up to 24 h (**Figure 2B**) or exposed to the indicated concentrations of CK for 24 h (**Figure 2C**). Our data showed that CK induces the release of cytochrome *c* from mitochondria to the cytosol and apoptosis-inducing factor (AIF) from the cytosol to the nucleus in time- and concentration-dependent manners. Caspase-3, poly(ADP-ribose)polymerase (PARP), and Bid, well-known downstream molecules of cytochrome *c*, were also cleaved in time- and concentration-dependent manners. In contrast, the expression of B-cell CLL/lymphoma 2 (Bcl2) protein, an anti-apoptotic molecule, decreased in time- and concentration-dependent manners. These data suggest that CK induces mitochondria-dependent apoptosis through cytochrome-*c*-dependent and -independent pathways.

CK Induces Apoptosis via AMPK Activation. Several reports demonstrated that activation of AMPK can lead to the induction of apoptosis in many human cancer cells (*13*−*15*). Therefore, we investigated whether phosphorylation of AMPK is induced by CK. HT-29 cells were treated with 50 μM CK for up to 24 h and were exposed with indicated concentrations of CK for 24 h. In comparison to the basal level (0 time), CK markedly stimulated the phosphorylation of AMPK in time- and concentration-dependent manners (panels **A** and **B** of **Figure 3**). No change in the expression of endogenous AMPKα was noted by immunoblotting with the AMPKα2 antibody. The time- and concentration-dependent phosphorylations of ACC, the main downstream protein of AMPK, ascertains activation of AMPK. If AMPK activation is associated with cytotoxic activity of CK, blocking AMPK activation with compound C, an AMPK inhibitor, should blunt the cytotoxic effect of CK. To confirm whether AMPK activation by CK is responsible for inducing apoptosis in cancer cells, HT-29 cells were treated with compound C (10−20 μM). Pretreatment of HT-29 cells with compound C significantly attenuated phosphorylations of AMPK and ACC in concentration-dependent manners (**Figure 3C**). As shown in **Figure 3D**, CK-induced apoptosis was completely abrogated by compound C. These results indicate that AMPK activation is associated with the induction of apoptosis.

AMPK Regulates the CK-Induced Apoptotic Signaling Pathway. Next, we examined whether CK-induced AMPK activation is associated with the expression of apoptosis proteins. As shown in panels **E** and **F** of **Figure 3**, CK-induced activation of AIF and cytochrome-*c*-dependent pro-apoptotic molecules, such as cytochrome *c*, caspase-3, caspase-8, caspase-9, and PARP, were significantly suppressed in the presence of compound C. In

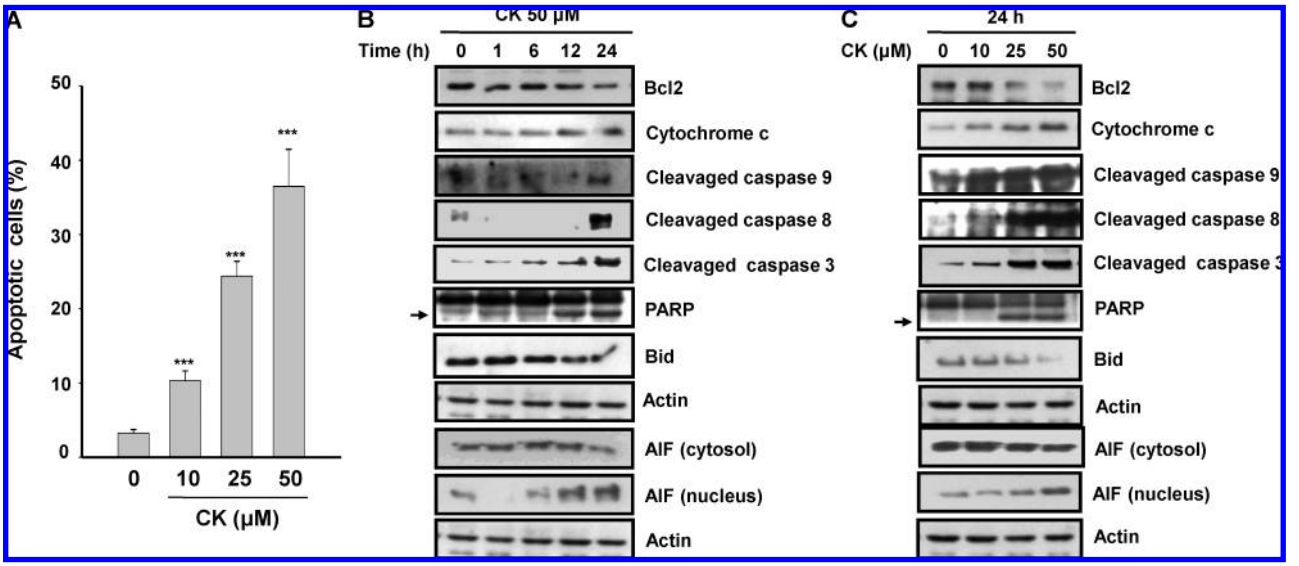

Figure 2. Effects of CK on cell death in HT-29 colon cancer cells. Cells were incubated with increasing concentration of CK for 24 h, then stained with propidium iodide, and analyzed for DNA content by flow cytometry. Data were obtained from 30 000 events and were represented as a percentage of cells in the sub-G_1 phase. Data were mean ± SE of three independent experiments (***, $p < 0.001$ versus control) (A). HT-29 cells were treated with 50 μM CK for indicated times (B) or different concentrations (10−50 μM) of CK for 24 h (C), and expression of apoptosis-related proteins were analyzed by Western blot using specific antibodies. The blot was reprobed with anti-actin antibody to confirm equal protein loading.

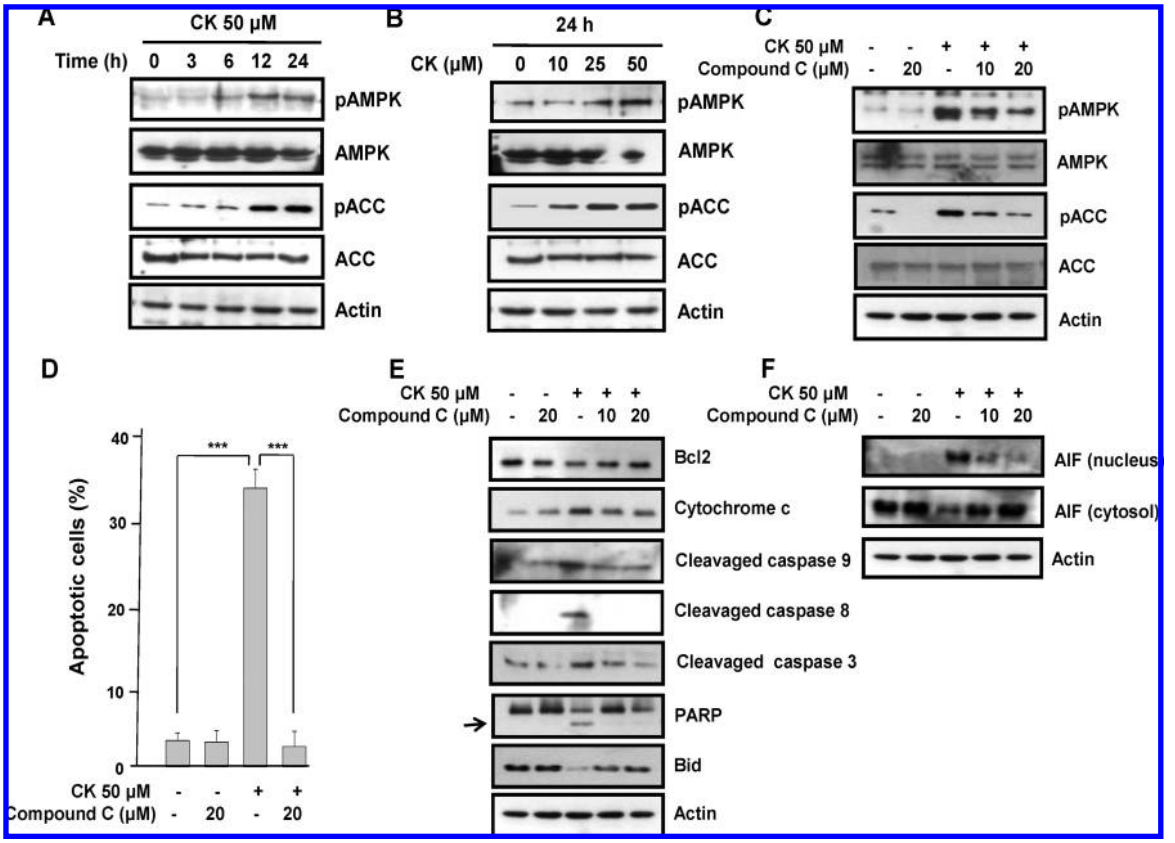

Figure 3. AMPK regulates CK-induced apoptosis in HT-29 cells. Cells were treated with 50 μM CK for indicated times (A) or different concentrations (10−50 μM) of CK for 24 h (B). After pre-incubation with compound C for 2 h, cells were treated with 50 μM CK for 24 h and expressions of apoptosis-related signal molecules were determined by Western blot. Actin was used as an internal control to evaluate the relative expression of protein (C, E, and F). Apoptosis was determined by fluorescence-activated cell sorting (FACS) analysis of DNA fragmentation of propidium-iodide-stained nuclei (D). Data were mean ± SE of three independent experiments (***, $p < 0.001$ versus control).

contrast, CK-induced decreases of Bcl2 and Bid protein expressions were reversed by treatment with compound C. To further confirm the role of AMPK in apoptosis, we knocked down AMPK in HT-29 cells using a siRNA. After transfection with siRNA for AMPK or control for 48 h, HT-29 cells were treated with 50 μM CK for 24 h. Transfection with AMPK siRNA

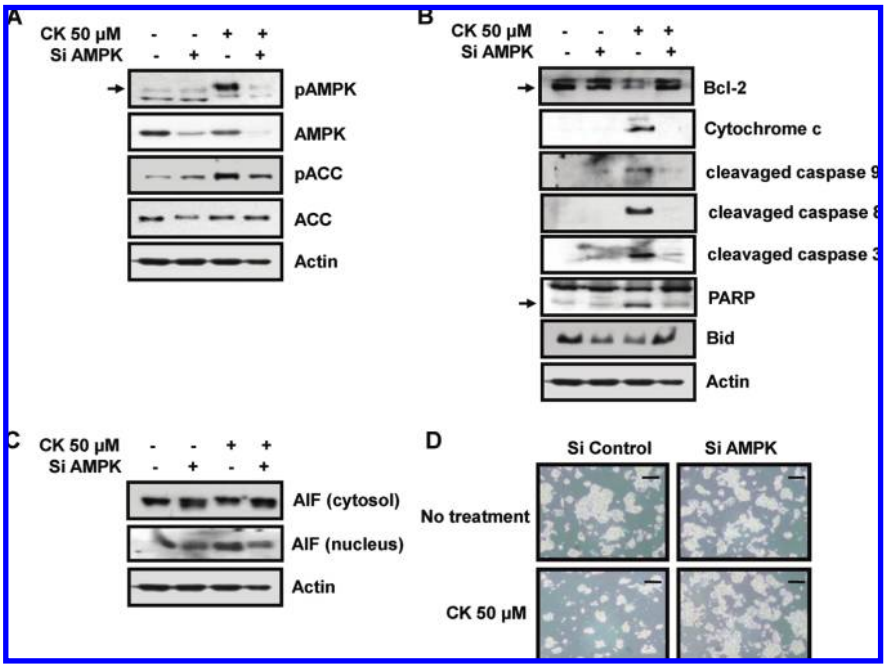

Figure 4. AMPK activation is necessary for CK-induced apoptotic cell death in HT-29 cells. HT-29 cells were transfected with either siRNA control or siRNA AMPK for 48 h and exposed to 50 μM CK for 24 h; then Western blots for pAMPK and pACC (A), cytochrome *c*-dependent proteins (B) or AIF (C) were performed; and cell viability was compared (D). The scale bar depicted in D is 100 μm.

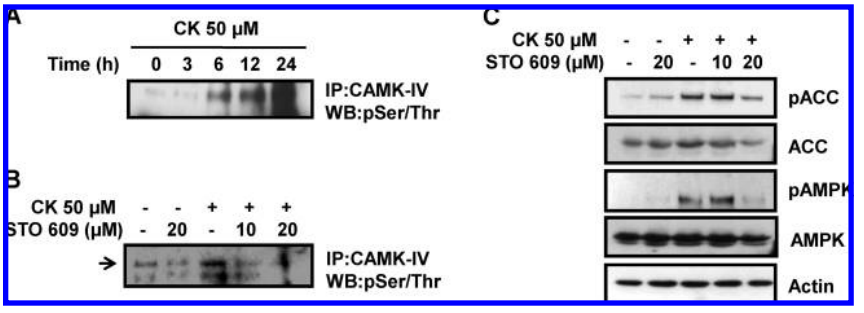

Figure 5. CAMK-IV is an upstream kinase for CK-induced AMPK phosphorylation in HT-29 cells. Cells were treated with 50 μM CK for indicated times (A) and pre-incubated with STO-609 for 2 h prior to stimulation with CK (B). Samples were immunoprecipitated with an antibody against CAMK-IV, and Thr^{172} phosphorylation was determined by Western blot. Phosphorylated forms of AMPK and ACC were concentration-dependently decreased in the presence of STO-609 (C).

significantly reduced AMPK expression compared to the control (**Figure 4A**). As expected, knockdown of AMPK expression by siRNA modulated apoptosis signals in caspase-dependent and -independent pathways (panels **B** and **C** of **Figure 4**). **Figure 4D** showed that AMPK knockout reduced the cytotoxic activity of CK. Therefore, we may conclude that CK induces mitochondria-mediated apoptosis via AMPK activation in HT-29 cells.

CAMK-IV Is Upstream of AMPK Activation. In recent years, it has been reported that both the tumor-suppressor LKB1 (*16*) and Ca^{2+}/calmodulin-activated protein kinase-IV (CAMK-IV) are AMPK kinases, which activate AMPK by directly phosphorylating Thr^{172} (*17*). To examine whether CK is able to activate LKB1 and/or CAMK-IV in HT-29 cells, the cells were exposed to 50 μM CK for 24 h and LKB1 and CAMK-IV protein expressions were analyzed by Western blot. As shown in panels **A** and **B** of **Figure 5**, HT-29 cells exhibited a time-dependent increase in CAMK-IV protein phosphorylation compared to control cells. However, there was no significant difference in LKB1 protein expression between CK-treated and control cells (data not shown). Next, we determined whether CAMK-IV activation is necessary for CK-induced AMPK phosphorylation using STO-609, CAMKs inhibitor. Pretreatment of HT-29 cells with STO-609 significantly attenuated phosphorylation of CAMK-IV in a concentration-dependent manner (**Figure 5B**), and inhibition of CAMK-IV by STO-609 abolished the phosphorylations of AMPK and ACC (**Figure 5C**).

DISCUSSION

Promising anticancer agents with strong inhibitory properties of survival-related proteins or activating capacities of apoptotic proteins are scrutinized for cancer treatments, especially those from natural origins because natural compounds are considered safe because they are derived from commonly consumed foodstuff. Ginseng radix, the root of *P. ginseng* C.A. Meyer, has been used as a traditional herbal medicine in Asian countries and is now widely used for preventive and therapeutic purposes. Recently, ginsenosides have been found to have many biological activities, including anti-inflammatory, anti-allergic, antidiabetic, and anticancer activities in cells or animals (*18*, *19*). However, several groups have proposed that this ginsenoside acts as a prodrug that is metabolized to the active form by intestinal

bacteria. CK is a metabolite of protopanaxadiol ginsenoside formed by intestinal bacteria. Although the anticancer effects of CK have been documented in various cancer cells (*19*, *20*), there were none on the apoptotic effect of CK in colon cancer cells associated with CAMK-IV/AMPK pathways.

Flow cytometric analyses revealed that CK increased the apoptotic sub-G_1 fraction in a concentration-dependent manner. However, there were no differences in the G_0/G_1 fraction when compared to the basal level. In addition, $p27^{KipI}$ and $p21^{WAF1/CIP1}$, which are associated with G_1 arrest, were not changed for up to 24 h (data not shown). These results suggest that CK exhibits antitumor activity through apoptosis induction rather than cell cycle arrest. The collapse of mitochondrial transmembrane potential mediates apoptosis, in that it allows the release of apoptotic mediators into the cytoplasm, such as cytochrome *c* and apoptosis-inducing factor (AIF). Cytochrome *c* normally resides in the mitochondrial inner membrane space and plays a central role in caspase-dependent cell death (*21*). Previous studies have already reported that CK plays an important role in triggering the mitochondria-mediated apoptosis pathway in different cancer cells in which caspases are the central components (*18*, *22*). As shown in **Figure 2**, CK perturbed the mitochondrial membrane of HT-29 cells, resulting in the release of cytochrome *c* and cleavage of pro-caspase-9, pro-caspase-8, pro-caspase-3, and PARP proteins, indicating caspase-dependent apoptosis. Bcl2 and its homologue Bcl-XL, both anti-apoptotic factors, usually have roles in preventing mitochondrial membrane disruption and the release of cytochrome *c* and other pro-apoptotic factors; meanwhile, Bax/Bak-like protein and Bid, pro-apoptotic factors, promote these events (*23*, *24*). In our study, CK downregulated anti-apoptotic Bcl2 protein expression, while Bax protein was not changed (data not shown). CK also induced the cleavage of Bid via activation of caspase-8. Truncated Bid (tBid) relocates to the mitochondria to further disturb the mitochondrial membrane; translocation of tBid to the mitochondria amplifies mitochondrial pathway activation (*25*).

CK also induced the release of AIF from mitochondria to the cytoplasm and translocation of AIF from the cytoplasm to the nucleus. AIF plays a central role in caspase-independent cell death, and AIF-mediated apoptosis has been observed in a large array of colon cancer cell lines. The interaction between AIF and cyclophlin A in the cytosol results in the DNAase activity of AIF (*26*). In the nucleus, AIF binds preferentially to single strands of DNA by electrostatic interactions between AIF (positively charged) and the negative charges of DNA. The key molecule of this cell death is PARP-1, which is a nuclear enzyme that is pivotal in the DNA damage network. When PARP-1 activation occurs, a PAR polymer is synthesized in the nucleus and released into the cytoplasm, where it leads to the release of AIF.

AMPK is a conserved serine/threonine protein kinase regulator of cellular metabolism that is activated in response to nutrient deprivation and pathological stress (*27*). Recent studies have revealed that AMPK inhibits cell growth and proliferation and also positively regulates apoptosis (*28*). However, its role in cell proliferation and cell survival remain poorly documented and somewhat controversial. Kim et al. recently reported that AMPK performs a function for protection against the cytotoxic effect of cisplatin, thereby implying that AMPK is one of the cellular factors determining the cellular sensitivity to cisplatin (*29*). In the present study, we have examined whether CK-induced apoptosis is associated with AMPK activation. We have demonstrated that CK stimulates AMPK activation in HT-29 cells. Consistently, the apoptotic effects of CK were completely abolished by inhibiting AMPK activity by either AMPK inhibitor (compound C) or siRNA for AMPK (**Figures 3** and **4**). These results suggest that AMPK activation is associated with CK-induced apoptosis and serves as a positive regulator for apoptosis. We have also observed that CK induces phosphorylation of CAMK-IV, the upstream regulator of AMPK, in a time-dependent manner and CAMKs inhibitor STO-609 inhibited CK-induced AMPK phosphorylation (**Figure 5C**).

In conclusion, the present study demonstrates that ginseng-derived CK reveals molecular events in HT-29 colon cancer cells, including inhibition of cell growth, induction of apoptosis, and activations of CAMK-IV and AMPK pathways. We have identified the CAMK-IV as one of the upstream regulators of AMPK and activation of AMPK as the key element in apoptosis induction caused by CK. These findings provide a molecular basis for the antiproliferation activity of CK, which seems to be a potential chemotherapeutic or chemopreventive agent.

ABBREVIATIONS USED

CK, compound K; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; PARP, poly(ADP-ribose)polymerase, Bcl-2, B-cell CLL/lymphoma 2; AIF, apoptosis-inducing factor; CAMK-IV, Ca^{2+}/calmodulin-activated protein kinase-IV.

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