Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 24 (2013) 1096–1104

Journal of Nutritional Biochemistry

20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol, a metabolite of ginseng, inhibits colon cancer growth by targeting TRPC channel-mediated calcium influx☆,☆☆

Jeong Ah Hwang[a,1], Mun Kyung Hwang[a,e,1], Yongwoo Jang[c], Eun Jung Lee[a], Jong-Eun Kim[e], Mi Hyun Oh[a], Dong Joo Shin[a], Semi Lim[a], Geun og Ji[d], Uhtaek Oh[c], Ann M. Bode[e], Zigang Dong[e], Ki Won Lee[a,b,*], Hyong Joo Lee[a,*]

[a]*WCU Biomodulation Program, Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea*
[b]*Advanced Institutes of Convergence Technology, Seoul National University, Suwon-si, Gyeonggi-do 443-270, Republic of Korea*
[c]*Department of Molecular Medicine, World Class University Program, Seoul National University, Seoul 151-921, Republic of Korea*
[d]*Department of Food Nutrition, Seoul National University, Seoul 151-921, Republic of Korea*
[e]*The Hormel Institute, University of Minnesota, Austin, MN 55912, USA*

Received 6 March 2012; received in revised form 18 July 2012; accepted 13 August 2012

Abstract

Abnormal regulation of Ca^{2+} mediates tumorigenesis and Ca^{2+} channels are reportedly deregulated in cancers, indicating that regulating Ca^{2+} signaling in cancer cells is considered as a promising strategy to treat cancer. However, little is known regarding the mechanism by which Ca^{2+} affects cancer cell death. Here, we show that 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20-GPPD), a metabolite of ginseng saponin, causes apoptosis of colon cancer cells through the induction of cytoplasmic Ca^{2+}. 20-GPPD decreased cell viability, increased annexin V-positive early apoptosis and induced sub-G1 accumulation and nuclear condensation of CT-26 murine colon cancer cells. Although 20-GPPD-induced activation of AMP-activated protein kinase (AMPK) played a key role in the apoptotic death of CT-26 cells, LKB1, a well-known upstream kinase of AMPK, was not involved in this activation. To identify the upstream target of 20-GPPD for activating AMPK, we examined the effect of Ca^{2+} on apoptosis of CT-26 cells. A calcium chelator recovered 20-GPPD-induced AMPK phosphorylation and CT-26 cell death. Confocal microscopy showed that 20-GPPD increased Ca^{2+} entry into CT-26 cells, whereas a transient receptor potential canonical (TRPC) blocker suppressed Ca^{2+} entry. When cells were treated with a TRPC blocker plus an endoplasmic reticulum (ER) calcium blocker, 20-GPPD-induced calcium influx was completely inhibited, suggesting that the ER calcium store, as well as TRPC, was involved. *In vivo* mouse CT-26 allografts showed that 20-GPPD significantly suppressed tumor growth, volume and weight in a dose-dependent manner. Collectively, 20-GPPD exerts potent anticarcinogenic effects on colon carcinogenesis by increasing Ca^{2+} influx, mainly through TRPC channels, and by targeting AMPK.
© 2013 Elsevier Inc. All rights reserved.

Keywords: Calcium influx; Colon cancer; 20-GPPD; TRPC

1. Introduction

Colon cancer is a major cause of cancer mortality in Western countries [1]. Half of all patients diagnosed with colorectal cancer eventually die from the disease, and less than 10% of patients with metastatic colorectal cancer survive more than 5 years after diagnosis [2]. Most cancer cells proliferate in an uncontrolled fashion and thus developing agents that trigger apoptotic cell death is a promising strategy to treat cancer [3]. Because colon cancer risk is related to nutritional factors, and numerous phytochemicals are reported to inhibit specific stages of carcinogenesis [4], natural compounds from foods could prove successful in preventing and treating colon cancer.

AMP-activated protein kinase (AMPK) is a sensor of energy balance at the cellular level. Once activated, AMPK switches off ATP-consuming pathways (e.g., fatty acid and cholesterol syntheses) and switches on ATP-generating pathways (e.g., fatty acid oxidation and glycolysis) [5]. AMPK regulates processes relevant to cancer devel-

☆ This work was supported by the Small and Medium Business Administration (S0807222-G0942940-10100013), World Class University Program (R31-10056) and World Class Institute Program founded by the Korea Research Foundation, Ministry of Education, Science and Technology. M.K.H. was supported by Seoul Science Fellowship.

☆☆ Conflicts of interest: None declared.

* Corresponding authors. Ki Won Lee is to be contacted at the Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea. Tel.: +82 2 880 4661; fax: +82 2 873 5095. Hyong Joo Lee, Major in Biomodulation, Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea. Tel.: +82 2 880 4853; fax: +82 2 873 5095.

E-mail addresses: kiwon@snu.ac.kr (K.W. Lee), leehyjo@snu.ac.kr (H.J. Lee).

[1] These authors contributed equally to this work.

0955-2863/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jnutbio.2012.08.008

opment, including cell cycle progression, tumor cell growth and cell survival [6]. At low energy levels, AMPK can be activated and phosphorylated by tumor suppressor kinase LKB1, as well as by Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) in response to an increase in cytosolic-free calcium $[Ca^{2+}]_c$ [7]. Ca^{2+} signaling is crucial in modifying and regulating most processes in healthy cells [8], particularly cellular homeostasis. Ca^{2+}-mediated signaling pathways are also involved in tumorigenesis, including metastasis, invasion and angiogenesis [9]. Thus, regulating Ca^{2+} signaling in cancer cells could be useful in cancer treatment. Thousands of Ca^{2+} channels exist, including store-operated, voltage-gated and transient receptor potential (TRP) channels. Among these, TRP channels, which are related to cancer progression, have been considered a potential target for anticancer strategies. The entry of Ca^{2+} into the cytoplasm through TRP channels expressed in cancer cells could induce a sustained high cytoplasmic Ca^{2+} concentration and kill cancer cells by causing apoptosis [10]. Mammalian TRP channels comprise six related protein families, which are putative six-transmembrane polypeptide subunits [11]. Among them, the transient receptor potential canonical (TRPC) channel functions as a receptor-operated channel that is stimulated by a receptor-phospholipase C (PLC)-activating cascade, typically by engaging a G protein-coupled receptor–Gq-PLCβ signaling pathway [12]. TRPC expression levels are altered in various cancers [13]. However, the expression and function of TRPC channels in colon cancer remain unclear.

Ginseng, one of the most widely used herbal medicines, is a therapeutic and pharmacological agent [14]. Ginsenosides, the unique active pharmacological compounds of ginseng, have many health effects, including anticancer, antidiabetic and antiaging properties [15]. After oral administration of ginsenosides, intestinal human bacteria transform protopanaxadiol (PPD)-type ginsenosides to 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20-GPPD) through the cleavage of sugar moieties [14]. 20-GPPD has been reported to exert antidiabetic [16,17], anti-inflammatory [18] and anticancer [19–21] effects. However, the molecular mechanisms and target(s) of 20-GPPD to induce apoptosis of colon cancer cells are poorly understood. In this study, we found that 20-GPPD induced apoptosis through Ca^{2+} influx through TRPC channels and subsequent activation of AMPK in CT-26 murine colon cancer cells. 20-GPPD also inhibited tumor growth in mouse allograft models.

2. Materials and methods

2.1. Chemicals

3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), fetal bovine serum (FBS), 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR), Gd^{3+}, SKF96365, 2-aminoethoxydiphenylborate (2-APB), U73122 and the antibody against β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Compound C, STO-609 and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate/acetomethyl (BAPTA/AM) were obtained from Calbiochem (San Diego, CA, USA). Gentamicin and L-glutamine were purchased from Life Technologies (Carlsbad, CA, USA). Fluo3-AM, Pluronic F-127 and annexin V-FITC kit were obtained from Invitrogen (Carlsbad, CA, USA). The antibodies against cleaved caspase-3, phosphorylated AMPK (Thr^{172}), total AMPK, phosphorylated ACC (Ser^{79}) and phosphorylated LKB1 (Ser^{428}) were purchased from Cell Signaling Technology (Beverly, MA, USA). Other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The AMPK kinase assay kits were obtained from Upstate Biotechnology (Lake Placid, NY, USA). [γ-^{32}P]ATP and the chemiluminescence detection kit were purchased from GE Healthcare (Piscataway, NJ, USA), and the protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Preparation of 20-GPPD

20-GPPD was produced from ginseng root extract, which was prepared by methanol extraction, using a thermostable recombinant β-glycosidase from *Sulfolobus solfataricus* [22]. The reaction solution was extracted with *n*-butanol, transferred to a clean tube, evaporated to dryness in a centrifugal evaporator (Eyela CVE-3100, Tokyo, Japan) and then reconstituted with methanol. The fraction of 20-GPPD was obtained by Prep-LC (Waters Delta Prep 4000, Waters, Milford, MA, USA) equipped with an Xbridge Prep C18 OBD column. The column was eluted with a 20:80 (v/v) mixture of acetonitrile and water. The flow rate was 1.0 ml/min. The fraction of 20-GPPD was evaporated to dryness in a centrifugal evaporator.

2.3. Cell culture

The CT-26 murine colon cancer cell line was obtained from the Korean Cell Lines Bank (Seoul, Korea). Cells were cultured in monolayers at 37°C in a 5% CO_2 incubator in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 2 mmol/L L-glutamine and 25 μg/ml gentamicin.

2.4. MTT assay

CT-26 cells were seeded (2×10^3 cells/well) onto 96-well plates. After different periods of culture, MTT solution (final concentration, 1 mg/ml) was added and the cells were then incubated for 20 min. Dark-blue formazan crystals that formed in intact cells were dissolved in dimethyl sulfoxide. Absorbance was measured at 570 nm using a microplate reader. The results were expressed as the percent of MTT reduction relative to the absorbance of untreated control cells.

2.5. DAPI staining assay

The DNA-specific fluorescent dye 4′6′-diamidino-2-phenylindole (DAPI) was used to detect nuclear fragmentation. CT-26 cells (2×10^5 cells/well in 24-well plates) were cultured for 24 h. Cells were treated with various concentrations of each compound for 24 h and then washed with PBS and stained with DAPI (1 μg/ml). After a 10-min incubation, the cells were observed under a fluorescence microscope (Olympus Optical, Tokyo, Japan).

2.6. Annexin V staining

CT-26 cells were grown and starved with serum-free media for 18 h. The cells were treated with 10 μM of 20-GPPD for 18 or 24 h, and early and late apoptotic/necrotic deaths were measured using the annexin V-FITC kit (Invitrogen). Briefly, cells were trypsinized and washed with serum-containing media and stained with annexin V conjugated with FITC and propidium iodide (PI) in binding buffer at room temperature for 5 min in the dark. Stained cells were analyzed by a flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA, USA).

2.7. Cell cycle analysis

The cell cycle was analyzed using flow cytometry as described previously [23], with slight modifications. CT-26 cells (2×10^5) were seeded onto a 10-cm dish and cultured for 24 h. The cells were treated with each compound for 24 h and then trypsinized, washed with ice-cold PBS and fixed with ice-cold 70% ethanol at −20°C overnight. Cells were then incubated with 20 μg/ml RNase A and 200 μg/ml PI in PBS at room temperature for 30 min in the dark and then subjected to flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson). The percentage of apoptotic cells in the sub-G1 peak was analyzed using the Cell Quest version 3.1f software (Becton Dickinson) and further analyzed with the ModFit (Verity Software House) or FlowJo (Tree Star, Inc.) program.

2.8. Western blot analysis

Cells (1×10^5) that had been cultured in a 6- or 10-cm dish for 48 h were starved in serum-free medium for an additional 18 h. The cells were then treated with various concentrations of each compound and Western blotting performed, as described previously [24].

2.9. In vitro AMPK kinase assays

In vitro AMPK kinase assays were performed in accordance with the manufacturer's instructions. In brief, each reaction solution contained 25 μl of assay reaction buffer [160 mM HEPES (pH 7.4), 3.25 mM dithiothreitol (DTT), 0.06% Brij-35] and a magnesium acetate-ATP cocktail buffer. For AMPK, 100 μM SAMS substrate peptide was included. Then, 2.5-μl aliquots containing 5 μl of each substrate and 10 μl diluted [γ-^{32}P] ATP solution were removed from the reaction mixture and incubated at 30°C for 10 min. Afterward, 15-μl aliquots were transferred onto p81 paper and washed three times with 0.75% phosphoric acid for 5 min per wash and once with acetone for 5 min. Radioactive incorporation was determined using a scintillation counter (LS6500; Beckman Coulter, Brea, CA, USA).

2.10. Measurement of intracellular Ca^{2+}

CT-26 cells were plated on a poly-D-lysine (PDL)-coated Lab-Tek 8 chamber (Nunc, Rochester, NY, USA). Cells were loaded with the fluorescent dye Fluo3-AM mixed with the same volume of 0.1% Pluronic F-127 and diluted in a normal bath solution (NBS) containing 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES (pH 7.4) to a final concentration of 5 μM. Cells were incubated at 37°C for 40 min after being loaded with Fluo3-AM. Then, cells were loaded with treatments in NBS and analyzed under a confocal microscope (UltraView ERS Rapid Confocal Imager; Perkin Elmer, Waltham, MA, USA).

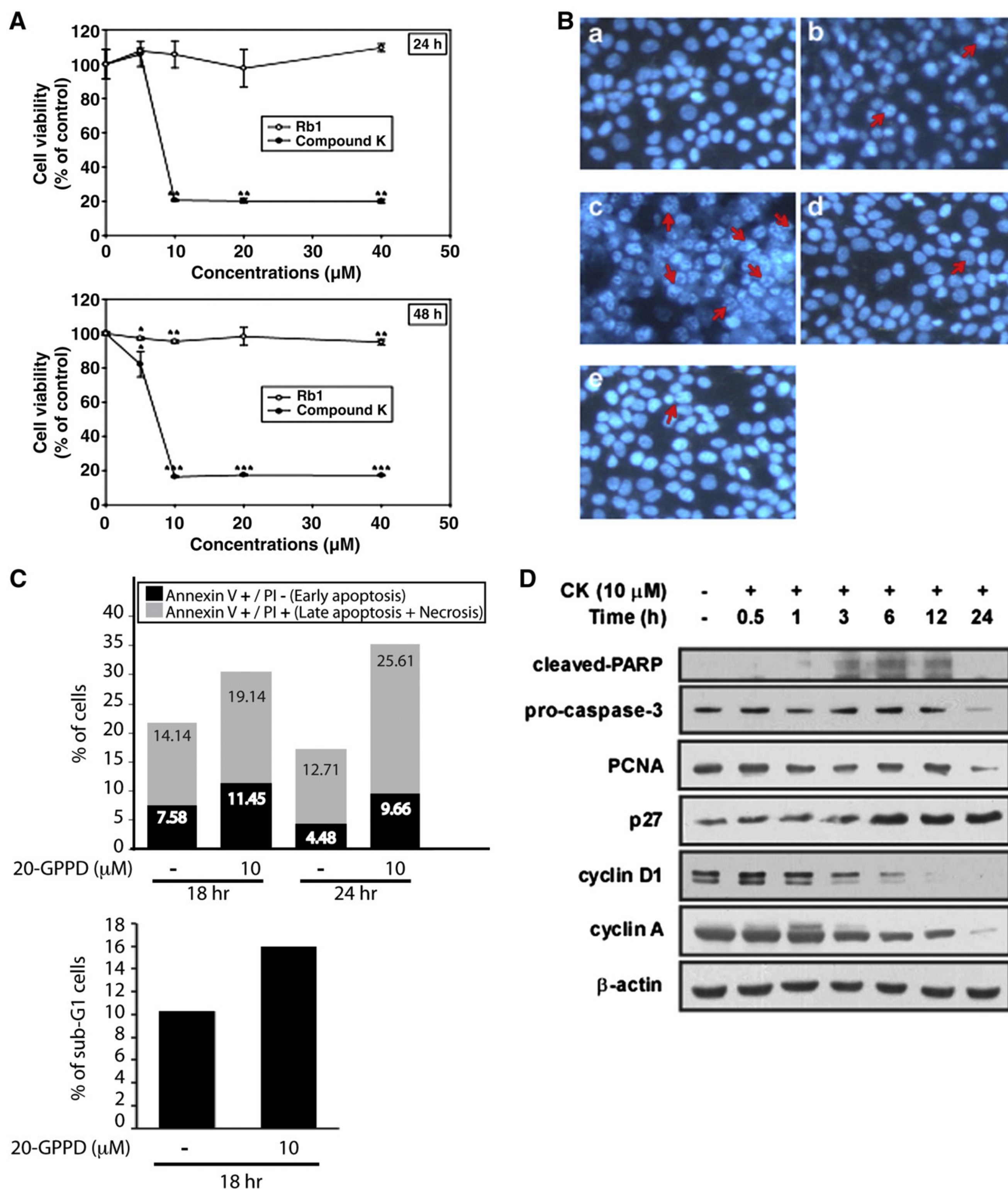

Fig. 1. Comparison of the apoptotic effects of 20-GPPD and ginsenoside Rb1. (A) 20-GPPD, but not ginsenoside Rb1, induces CT-26 cell death. Cells were starved for 18 h and then treated with 20-GPPD or ginsenoside Rb1 (5, 10, 20 or 40 μM) for the indicated times. Cell viability was determined by MTT assay, as described in Materials and Methods. (B) 20-GPPD, but not ginsenoside Rb1, induces apoptotic cell death. Nuclear condensation and apoptotic bodies are shown in cells stained with DAPI and examined under a fluorescence microscope. The cells were starved for 18 h and then treated with each compound for 24 h: (a) no treatment; (b) 5 μM 20-GPPD; (c) 10 μM 20-GPPD; (d) 5 μM ginsenoside Rb1; (e) 10 μM ginsenoside Rb1. (C) 20-GPPD induces apoptotic death (upper panel) and arrests cell cycle in sub-G1 phase (lower panel). Cells were starved for 18 h and then treated with each compound for the indicated times. Early apoptotic death was determined by measuring annexin V-positive and PI-negative cells. Late apoptotic and necrotic death was determined by measuring annexin V-positive and PI-positive cells. Sub-G1 fractions were determined by PI staining and further analysis with the FlowJo program. (D) 20-GPPD induced apoptotic signals. The cells were starved for 18 h, treated with 20-GPPD (10 μM) for the indicated times and the protein levels were determined by Western blot analysis. Data are representative of three independent experiments with similar results.

2.11. CT-26 Cell tumor allograft in mice

Female BALB/c mice (5 weeks old; mean body weight, 25 g) were purchased from the Institute of Laboratory Animal Resources, Seoul National University, Seoul, Korea. Animals were acclimated 1 week prior to the experiment and had free access to food and water. The animals were housed in climate-controlled quarters (24°C, 50% humidity) on a 12-h light/12-h dark cycle. The experimental protocols were approved by the Animal Care and Use Committee of Seoul National University. CT-26 cells (1.5×10^5 cells) were inoculated subcutaneously in the left flank of each mouse. The animals were randomized into control and treatment groups (seven mice per group). Treatment was initiated when the tumors reached a mean volume of 150 mm^3. The first treatment day was designated Day 0. For injections, 20-GPPD was dissolved in 20% ethanol and injected intraperitoneally daily at 0.2, 0.5 or 1 mg/kg (100 μl/mouse). The control group received 20% ethanol only (100 μl/mouse). Tumor volume (V), measured using an external caliper once a week, was calculated as $V=0.5238\times\text{length}\times\text{width}\times\text{height}$. The treatment was continued until a tumor reached ~2000 mm^3 in volume, at which time the animal was euthanized and the tumor removed.

3. Results

3.1. 20-GPPD, but not ginsenoside Rb1, induces apoptosis of CT-26 murine colon cancer cells

To determine the possible cancer therapeutic effects of 20-GPPD, we investigated the effect of 20-GPPD on apoptosis in CT-26 cells. Treatment of CT-26 cells with 20-GPPD, but not ginsenoside Rb1, markedly reduced cell viability (Fig. 1A). To further determine whether the decrease in cell viability was caused by the apoptotic cell death, we used DAPI nuclear staining and observed that 20-GPPD, not ginsenoside Rb1, induced chromatin condensation and nuclear fragmentation, which are representative markers of apoptosis, in a dose-dependent manner (Fig. 1B). Since the cell viability dropped severely after treatment of 20-GPPD only at 10 μM, we next confirm whether 20-GPPD also mediates other types of cell death than apoptosis using annexin V and PI counter staining. The result showed that early apoptotic deaths are increased more than 1.5- and 2-fold after being treated with 10 μM 20-GPPD at 18 and 24 h, respectively (Fig. 1C; upper panel). However, late apoptotic and necrotic deaths are also increased 1.3- and 2-fold after being treated with 10 μM 20-GPPD at 18 and 24 h, respectively (Fig. 1C; upper panel). 20-GPPD induces early apoptotic death, but it also significantly increased late apoptotic and necrotic death. To determine whether the decrease of cell viability was also associated with cell cycle arrest, we analyzed cell cycle distribution using flow cytometry. 20-GPPD-treated cells were significantly converged in the sub-G1 phase, compared to the untreated control group (Fig. 1C; lower panel). In addition to sub-G1 phase accumulation, however, the cell cycle distribution shows the

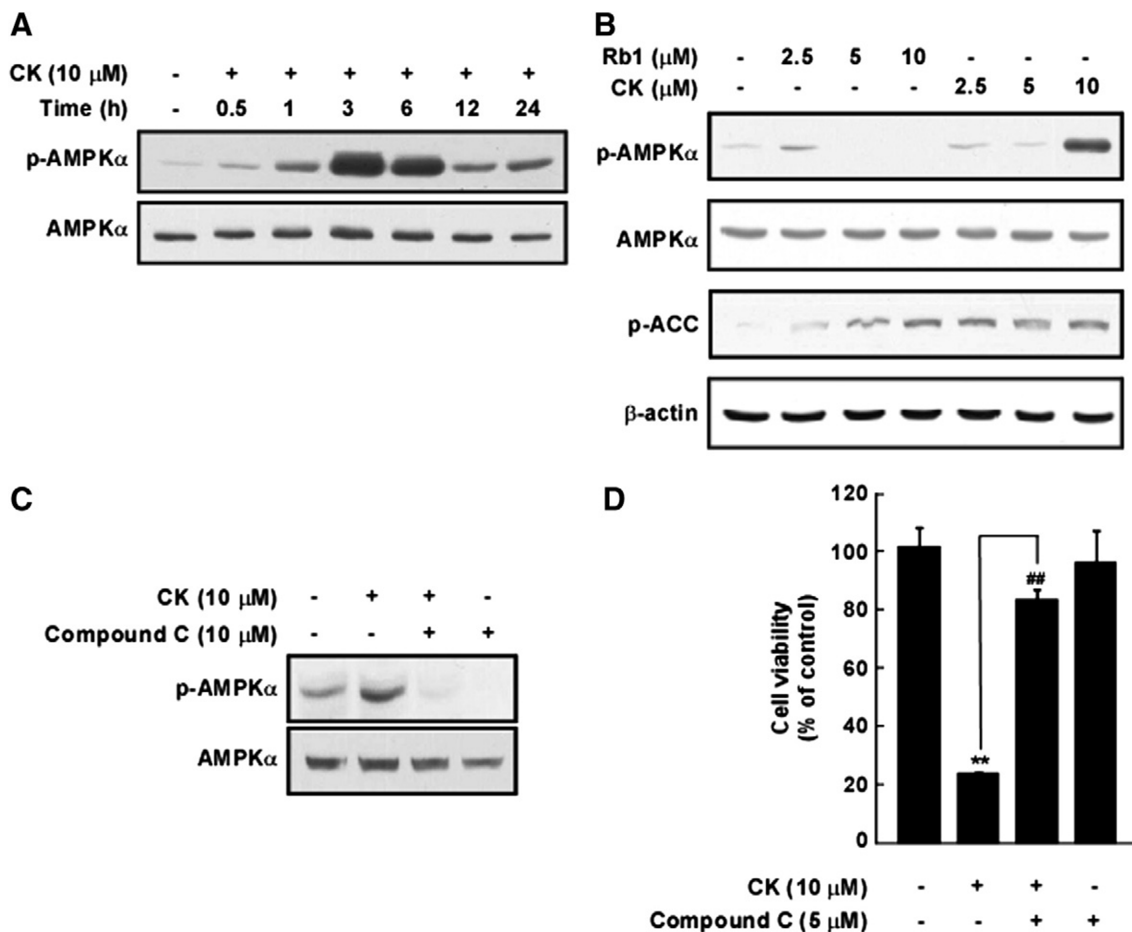

Fig. 2. Effect of 20-GPPD on AMPK activation in CT-26 cells. (A) 20-GPPD induces AMPK phosphorylation in a time-dependent manner. (B) 20-GPPD, but not ginsenoside Rb1, induces phosphorylations of AMPK and ACC in a dose-dependent manner. For (A) and (B), cells were starved for 18 h and then treated with 20-GPPD or ginsenoside Rb1. After 6 h, the levels of each protein were determined by Western blot analysis as described in Materials and Methods. (C) Compound C, an AMPK-specific inhibitor, suppresses 20-GPPD-induced AMPK phosphorylation. Cells were starved for 18 h and pretreated with compound C (10 μM). After 1 h, the cells were treated with 20-GPPD (10 μM) and incubated for 6 h. The levels of phosphorylated and total AMPK were then determined by Western blot analysis. (D) Compound C protects cells from 20-GPPD-induced cell death. The cells were starved for 18 h and then pretreated with compound C (10 μM). After 1 h, the cells were treated with 20-GPPD (10 μM) and incubated for 24 h. Cell viability was then determined by MTT assay. Asterisks indicate a significant difference (**$P<.01$) compared to untreated control, and pound signs indicate a significant difference (##$P<.01$) compared to the 20-GPPD-treated group. Data are representative of three independent experiments with similar results.

loss of G2 peak and the broad accumulation of cells through the G1 and S-phase without any obvious boundary between them, only after treatment with 20-GPPD (data not shown). These data might imply that 20-GPPD might be involved in other phases of cell cycle arrest than sub-G1 arrest. Furthermore, 20-GPPD increased PARP cleavage and p27 expression, but attenuated procaspase-3, PCNA, cyclin D1 and cyclin A expression levels (Fig. 1D). Collectively, these results demonstrated that 20-GPPD induces CT-26 cellular apoptosis.

3.2. 20-GPPD induces apoptosis of CT-26 murine colon cancer cells mediated through AMPK phosphorylation

AMPK and acetyl-CoA carboxylase (ACC), a downstream effector of AMPK, have been reported to induce cancer cell death [25]. To investigate the possibility that AMPK plays a key role in inducing apoptosis by 20-GPPD, we examined the effects of 20-GPPD and ginsenoside Rb1 on the phosphorylation of AMPK and ACC in CT-26 cells. Western blot data showed that 20-GPPD increased AMPK phosphorylation in a time-dependent manner (Fig. 2A), and this effect of 20-GPPD was more potent than that of ginsenoside Rb1 (Fig. 2B). To examine the role of 20-GPPD on AMPK activation, we used compound C, a synthetic AMPK inhibitor, and found that compound C completely suppressed 20-GPPD-induced AMPK phosphorylation (Fig. 2C). Moreover, compound C recovered the 20-GPPD-induced reduction in cell viability (Fig. 2D), indicating that 20-GPPD induces apoptosis of CT-26 cells by activating AMPK.

3.3. AMPK is involved in the apoptotic death of CT-26 murine colon cancer cells

To determine whether AMPK activation caused CT-26 cellular apoptosis, we used AICAR, a cell permeable AMPK activator. Western blot analysis showed that AICAR induced phosphorylation of AMPK at 1 h (Supplemental Fig. 2A) and decreased CT-26 cell viability in a dose-dependent manner (Supplemental Fig. 2B). Similar to 20-GPPD, AICAR also induced PARP and caspase-3 cleavages (Supplemental Fig. 2C). Thus, CT-26 cellular apoptosis is associated with AMPK activation.

3.4. 20-GPPD does not directly affect AMPK or LKB1 activity

To determine whether 20-GPPD directly induced AMPK activity, we conducted an *in vitro* AMPK kinase assay, which showed that 20-GPPD did not induce AMPK kinase activity (Fig. 3A), suggesting that 20-GPPD-induced AMPK phosphorylation is not regulated through direct activation but rather through the modulation of upstream signals. Because LKB1, a tumor suppressor, is a well-known upstream kinase of AMPK [26], we investigated whether 20-GPPD induced LKB1 activation, but found no alteration in LKB1 phosphorylation stimulated by 20-GPPD (Fig. 3B). Thus, 20-GPPD-induced AMPK phosphorylation is regulated by upstream signals other than LKB1.

3.5. Intracellular Ca^{2+} levels regulate 20-GPPD-induced AMPK phosphorylation and apoptosis in CT-26 murine colon cancer cells

Because Ca^{2+} has been reported to activate AMPK [27], we next investigated whether an increase in the cytosolic Ca^{2+} level could result in AMPK phosphorylation in CT-26 cells. Western blot analysis showed that an increased Ca^{2+} concentration was associated with increased AMPK phosphorylation (Fig. 4A). BABTA/AM, a Ca^{2+} chelator, completely suppressed 20-GPPD-induced phosphorylation of AMPK (Fig. 4B) and cell death (Fig. 4C), indicating that Ca^{2+} is involved in the 20-GPPD-induced apoptosis through AMPK activation. CaMKKβ is an upstream kinase of AMPK that senses cytosolic Ca^{2+} levels [28]. Consistent with these results, STO-609, an inhibitor of CaMKKβ, completely suppressed AMPK phosphorylation induced by 20-GPPD (Fig. 4D). Collectively, these results suggest that increased levels of Ca^{2+} influx caused by 20-GPPD play a key role in AMPK activation and subsequent apoptosis of CT-26 cells.

3.6. 20-GPPD increases Ca^{2+} influx through TRPC channels

The increased expression of some TRP channels, which are calcium selective, is associated with colon cancer [10]. Therefore, we investigated whether increases in Ca^{2+} in response to 20-GPPD were due to TRP channel activation. Using Fluo-3 fluorescent images, we found that 20-GPPD increased Ca^{2+} entry into CT-26 cells in a dose-dependent manner (Fig. 5A, Supplemental movie S3), which was blocked by ruthenium red, a broad TRP channel blocker (Fig. 5B), indicating that TRP channel activation induces the entry of extracellular Ca^{2+}. To further determine which TRP channels were involved, we treated cells with capsazepine, a TRPV1 blocker. Although the increased expression of TRPV1 has been reported to be associated with colon cancer [29], capsazepine had no effect on the Ca^{2+} influx induced by 20-GPPD (Fig. 5B). Because Ca^{2+} entry through TRPC channels is associated with AMPK activation [30], we next treated cells with TRPC blockers and found that Gd^{3+} and SKF96365, TRPC blockers, reduced the increased Ca^{2+} influx induced by 20-GPPD (Fig. 5C and D). TRPC channels are store-operated Ca^{2+} channels, with Ca^{2+} concentration regulated by channels in the endoplasmic reticulum (ER). To test whether ER channels were involved in the Ca^{2+} increase induced by 20-GPPD, we treated cells with 2-APB, an ER channel blocker, in the presence of TRPC blockers. Co-treatment with 2-APB and Gd^{3+} or SKF96365 more strongly blocked the Ca^{2+} influx increase caused by 20-GPPD compared to Gd^{3+} or SKF96365 alone (Fig. 5C and D, Supplemental movie S4), indicating that Ca^{2+}

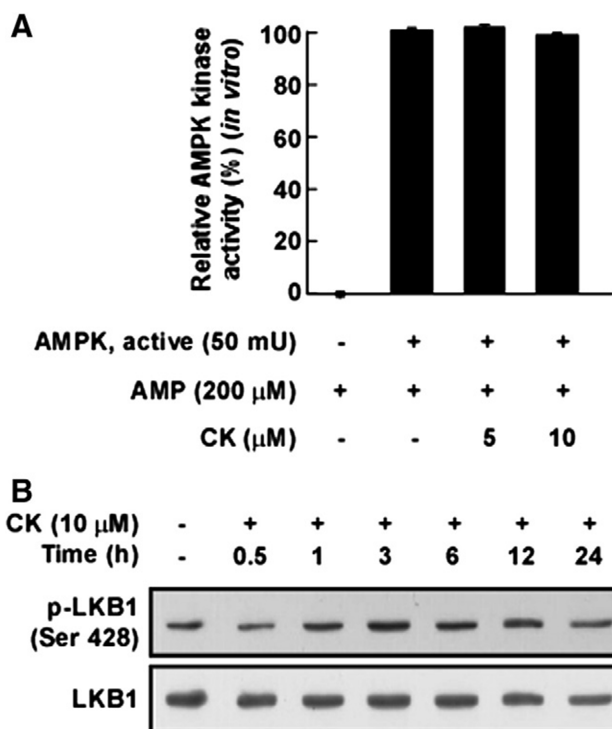

Fig. 3. LKB1, an upstream kinase of AMPK, does not affect 20-GPPD-induced AMPK phosphorylation. (A) 20-GPPD does not directly increase AMPK kinase activity *in vitro*. AMPK kinase assays were performed as described in Materials and Methods. (B) 20-GPPD does not induce the phosphorylation of LKB1. Cells were starved for 18 h and then treated with 20-GPPD (10 μM) for the indicated times. The protein levels were determined by Western blot analysis. Data are representative of three independent experiments with similar results.

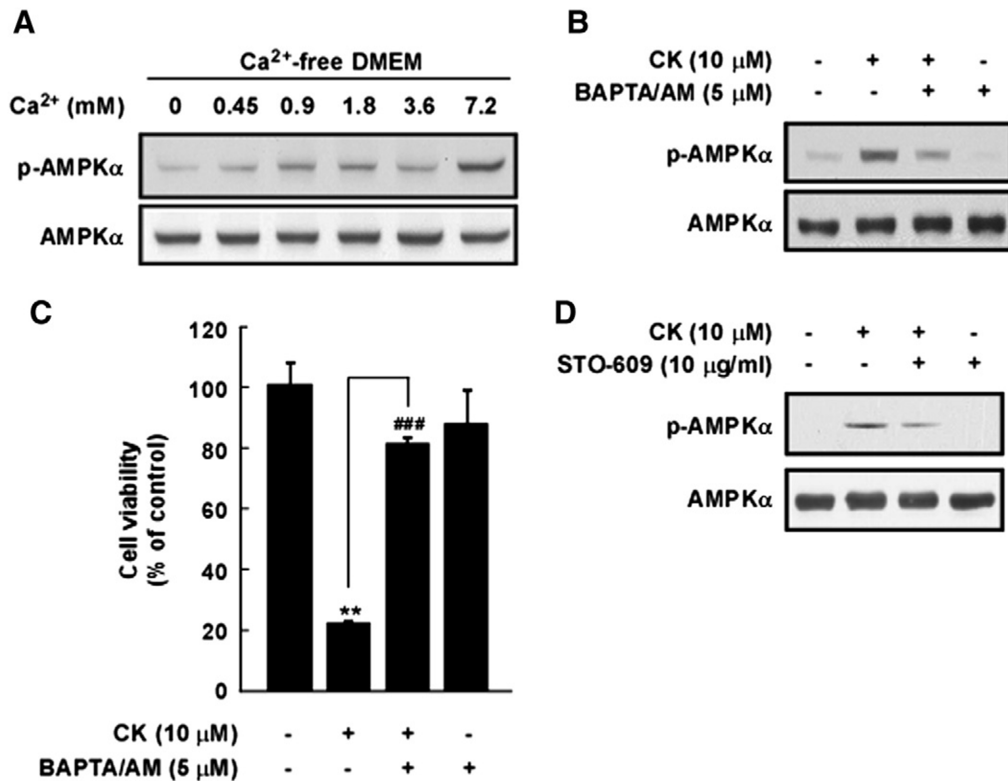

Fig. 4. Effect of Ca^{2+} influx on AMPK phosphorylation and cell death. (A) Ca^{2+} influx increased AMPK phosphorylation in a dose-dependent manner. CT-26 cells were starved for 18 h and then treated with Ca^{2+} ($CaCl_2$ in Ca^{2+}-free DMEM) at the indicated concentrations. After 6 h, the levels of phosphorylated and total AMPK were determined by Western blot analysis. (B and C) BAPTA/AM, a Ca^{2+} chelator, suppresses 20-GPPD-induced AMPK phosphorylation and cell death. The cells were starved for 18 h and then pretreated with BAPTA/AM (5 μM). After 1 h, the cells were treated with 20-GPPD (10 μM) and incubated for 6 (B) or 24 h (C). Protein levels were measured by Western blot analysis and cell viability was determined by MTT assay. Asterisks indicate a significant difference (**P<.01) compared to control, and pound signs indicate a significant difference (###P<.001) compared to the 20-GPPD-treated group. (D) STO-609, a CaMKKβ inhibitor, suppresses 20-GPPD-induced AMPK phosphorylation. The cells were starved for 18 h and then pretreated with STO-609. After 1 h, the cells were treated with 20-GPPD (10 μM) for 6 h, after which the protein levels were determined by Western blot analysis. Data are representative of three independent experiments with similar results.

release from the ER is also involved. To examine whether 20-GPPD affected only the ER channels, we treated cells with 2-APB or U73122, a PLC inhibitor. However, neither compound completely blocked Ca^{2+} entry induced by 20-GPPD (data not shown), indicating that 20-GPPD affects Ca^{2+} influx by modulating TRPC channels, together with Ca^{2+} release from the ER.

3.7. 20-GPPD inhibits tumor growth of colon cancer cells in a mouse allograft model

To determine whether 20-GPPD had similar effects in *in vivo* carcinogenesis models, we conducted an animal experiment using a mouse allograft model of injected CT-26 cells. The tumor volume of the 20-GPPD-treated group was significantly suppressed (Fig. 6A). The average tumor volume of mice in the untreated group increased, reaching 2104 mm^3 at 38 days postinoculation, whereas the average tumor volumes of mice treated with 0.2, 0.5 or 1 mg/kg 20-GPPD were 1428, 950 or 822 mm^3, respectively (Fig. 6B). In addition, treatment with 20-GPPD notably reduced tumor weight compared to tumor weights in the control group (Fig. 6C). The body weights of mice in the control and 20-GPPD-treated groups did not differ significantly, indicating that the dosages used were not toxic to the animals (data not shown).

4. Discussion

20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (20-GPPD) is an intestinal metabolite of protopanaxadiol-type saponins, such as ginsenoside Rb1, Rb2 and Rc (Supplemental Fig. 1). The glycosylated form of ginsenosides is not well absorbed in the stomach because of its polarity. Thus, the less glycosylated the ginsenoside is, the more effective it is in cancer prevention [31]. The anticarcinogenic effects of 20-GPPD appear to stem mainly from its induction of apoptotic death of cancer cells, including astrocytoma [20], B16-BL6 melanoma [32] and HL-60 leukemia [33,34]. However, the molecular mechanism and target(s) of 20-GPPD in colon cancer apoptosis are poorly understood.

Cellular stresses, including metabolic stresses such as glucose deprivation, hypoxia and oxidative stress [35], first activate AMPK and increase the AMP/ATP ratio. Findings that AMPK activation results in increased fatty acid oxidation and glucose uptake focused attention on treating metabolic diseases, such as obesity and diabetes, through AMPK activation [36]. However, many proteins involved in cell cycle and protein synthesis, such as mTOR and p53, are AMPK substrates, implying that AMPK may have a role in carcinogenesis [6]. Metformin, which activates AMP and is widely used as a diabetes drug, and AICAR, another well-known AMPK activator, have been investigated as potential new anticancer agents that inhibit cancer cell growth [37,38]. In this study, the apoptotic effect of 20-GPPD on CT-26 cells involved AMPK activation. Recently, AMPK activation induced by 20-GPPD has been reported in other cellular systems, including HepG2 human hepatoma cells [16] and 3T3-L1 adipocytes [39]. In HT-29 human colon cancer cells, 20-GPPD is also reported to activate AMPK and induce apoptosis [40], although the primary target of 20-GPPD in AMPK activation remains unclear.

The tumor suppressor LKB1 is the first identified mammalian upstream AMPK kinase and reduces the direct connection of

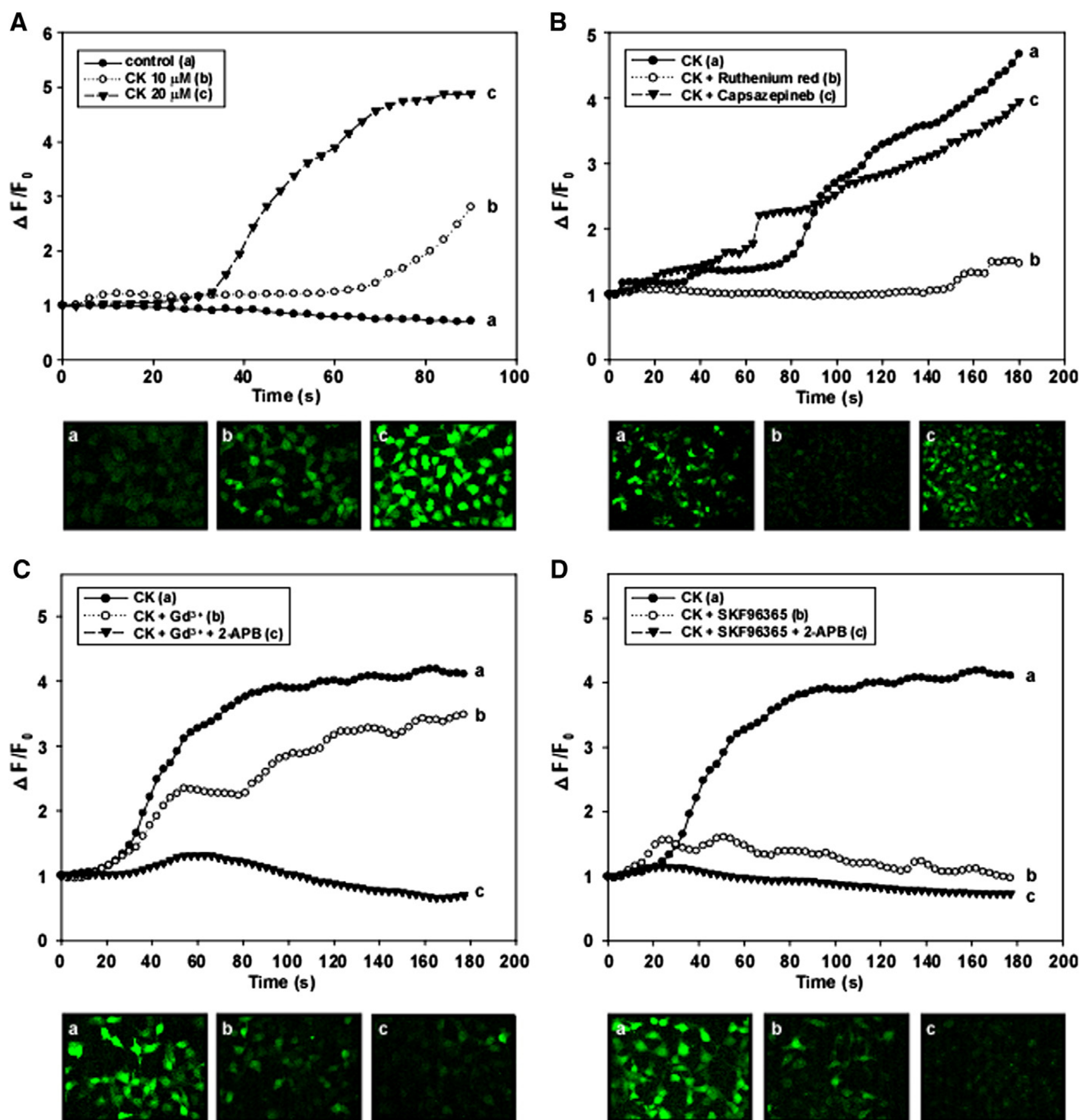

Fig. 5. Involvement of TRPC channels in 20-GPPD-induced Ca^{2+} influx. (A) 20-GPPD (10 or 20 μM) increases Ca^{2+} entry into CT-26 cells in a dose-dependent manner. (B) Treatment with 5 μM ruthenium red, a TRP channel blocker, prevented 20-GPPD-induced Ca^{2+} influx, but 20 μM capsazepine, a TRPV1 antagonist, had no effect. (C and D) Effects of TRPC blockers (100 μM Gd^{3+} and 10 μM SKF96365) and ER blocker (100 μM 2-APB) on 20-GPPD-induced Ca^{2+} influx. After CT-26 cells were stained with Fluo-3 AM, Ca^{2+} entry was measured every 3 s using a confocal microscope. Cells to calculate fluorescence intensity were selected using the ImageJ software program. Each photo shows images of Ca^{2+} at the final time, and the letters 'a', 'b' and 'c' denote each group indicated in the graph.

metabolism to cancer mediated by AMPK [26]. However, the finding that AMPK has a significant activity in LKB1-null cells indicates that other upstream kinases that activate AMPK exist, among which is CaMKK [28]. Several AMPK activators were reported to activate AMPK through the activation of CaMKK, even in the absence of LKB1 [41,42]. In our study, 20-GPPD did not directly induce either AMPK or LKB1 activity. However, a CaMKK inhibitor blocked 20-GPPD-induced AMPK activation, indicating that CaMKK is involved in 20-GPPD-induced AMPK activation. Because most cancer cells have mutated forms of tumor suppressor proteins, the regulation of CaMKK, instead of LKB1, by AMPK activators could be important in treating cancers.

CaMKK is induced by an increase in intracellular Ca^{2+} levels. Hence, investigating the mechanisms of Ca^{2+} entry into the cytosol is crucial in determining the target protein of 20-GPPD that induces CaMKK-dependent AMPK activation. In our study, intracellular Ca^{2+}

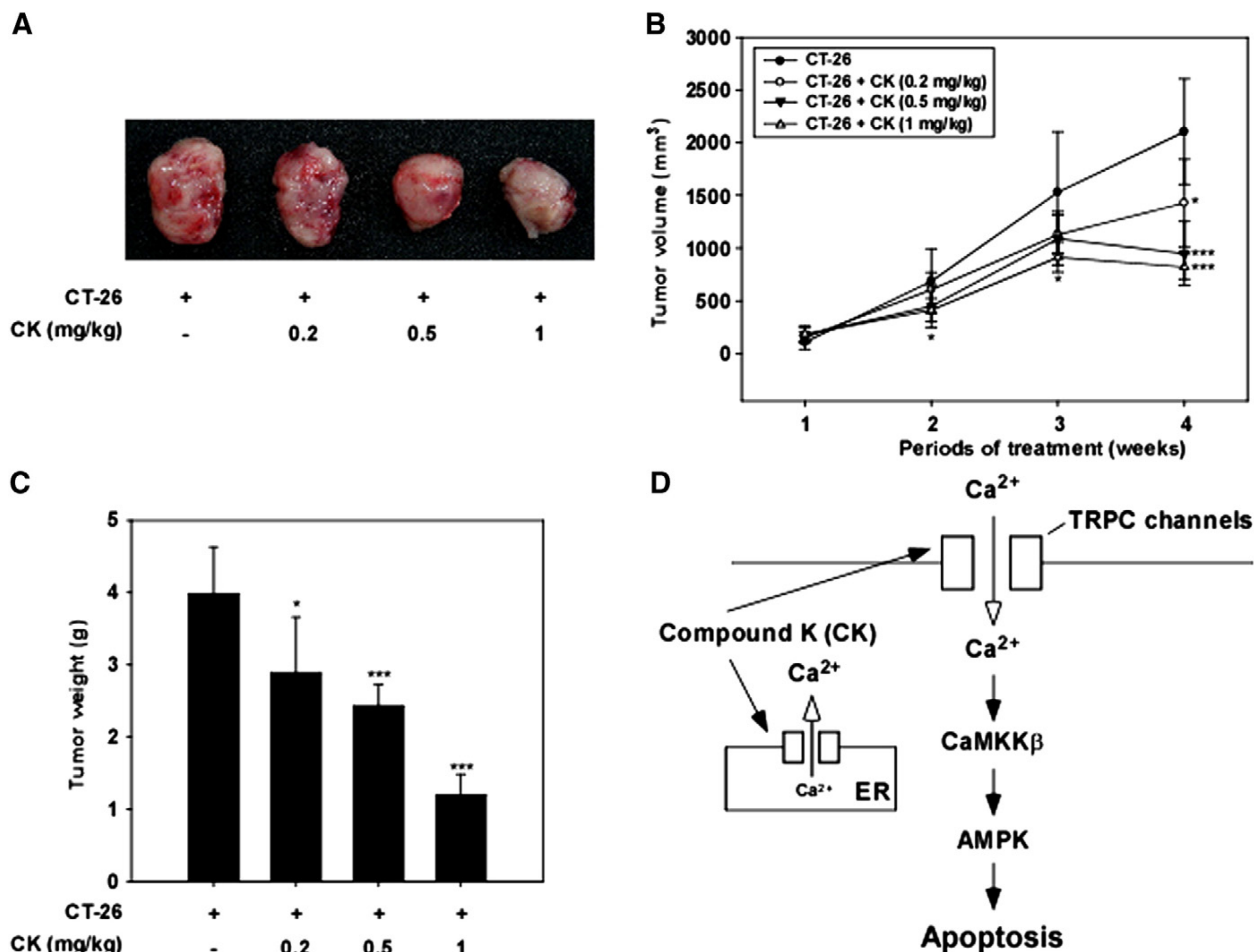

Fig. 6. 20-GPPD reduced colon tumors in an *in vivo* allograft model. Animals were treated as described in Materials and Methods. On Day 38, tumors were removed and subjected to further analyses. (A) Photographs of removed tumors. (B) The average tumor volumes of each group were plotted over 38 days after tumor cell injection. Asterisks indicate statistical significance of tumor growth inhibition by 20-GPPD (*P<.05; ***P<.001) compared to the untreated control group. (C) At the end of the experiment, the tumors from each group were weighed (n=7). Asterisks indicate a significant decrease in tumor weight between mice treated with 20-GPPD (*P<.05; ***P<.001) and untreated control. (D) Proposed mechanisms of 20-GPPD-induced apoptosis of colon cancer.

was induced by 20-GPPD, and a cell-permeable calcium chelator protected 20-GPPD-induced cell death and suppressed 20-GPPD-induced phosphorylation of AMPK. One hypothesis is that Ca^{2+} ionophores activate ATP-driven calcium pumps, leading to Ca^{2+} entry into cells and resulting in ATP depletion, which activates AMPK as a secondary consequence [28]. However, more detailed mechanisms of Ca^{2+} entry have not been elucidated. Ca^{2+} entry through TRPC channels is reported to be necessary for thrombin-induced NF-κB activation in endothelial cells by AMPK and protein kinase Cδ [30]. In addition, the induction of TRPC1 expression is reported to sensitize intestinal epithelial cells to apoptosis [43]. Here, we demonstrated that Ca^{2+} entry through TRPC channels leads to AMPK activation and induces apoptosis in colon cancer.

Mammalian TRP channels are divided into seven families. Among them, TRPC is a putative store-operated Ca^{2+} channel (SOC) that links slow and sustained extracellular Ca^{2+} entry to rapid and transient Ca^{2+} release from ER stores [44]. Although Ca^{2+} is double edged, in that it is involved in both cell proliferation and death, ER-mediated Ca^{2+} overload has been linked directly to caspase activation and apoptosis [45]. Thus, we examined TRPC- and ER-mediated changes in Ca^{2+} levels as an upstream regulator of 20-GPPD-induced apoptosis. TRPC channel blockers attenuated Ca^{2+} entry induced by 20-GPPD into CT-26 cells. However, these blockers did not completely suppress 20-GPPD-induced Ca^{2+} entry, and the fluorescent phenotype flickered as time passed. Because Ca^{2+} oscillation is the major feature of ER stores and SOC blockage triggers ER Ca^{2+} release, these results indicate that Ca^{2+} release from the ER is involved in the 20-GPPD-induced increase of intracellular Ca^{2+}, as is the TRPC. This is the first report revealing the involvement of TRPC and ER calcium channels in colon cancer. However, little is known about the role of TRPC in colon cancer development, and its involvement should be further investigated.

Finally, an *in vivo* experiment demonstrated that 20-GPPD suppressed tumor growth in mice. Thus, our evaluation of the anticancer effect of 20-GPPD on colon cancer showed that 20-GPPD induces apoptosis of CT-26 murine colon cancer cells *in vitro* and suppresses tumor growth *in vivo*. Our study also indicates that Ca^{2+} entry through TRPC channels is a novel molecular target of 20-GPPD that induces apoptosis of colon cancer cells (Fig. 6D). These results suggest that TRPC activation might be a promising strategy to treat colon cancers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.08.008.

References

[1] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225-49.
[2] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71-96.
[3] Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin 2005;55:178-94.
[4] Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3:768-80.
[5] Carling D. The AMP-activated protein kinase cascade–a unifying system for energy control. Trends Biochem Sci 2004;29:18-24.
[6] Fay JR, Steele V, Crowell JA. Energy homeostasis and cancer prevention: the AMP-activated protein kinase. Cancer Prev Res (Phila Pa) 2009;2:301-9.
[7] Hoyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T, et al. Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol Cell 2007;25:193-205.
[8] Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 2000;1:11-21.
[9] Monteith GR, McAndrew D, Faddy HM, Roberts-Thomson SJ. Calcium and cancer: targeting Ca^{2+} transport. Nat Rev Cancer 2007;7:519-30.
[10] Prevarskaya N, Zhang L, Barritt G. TRP channels in cancer. Biochim Biophys Acta 2007;1772:937-46.
[11] Clapham DE. TRP channels as cellular sensors. Nature 2003;426:517-24.
[12] Birnbaumer L. The TRPC class of ion channels: a critical review of their roles in slow, sustained increases in intracellular Ca(2+) concentrations. Annu Rev Pharmacol Toxicol 2009;49:395-426.
[13] Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential cation channels in disease. Physiol Rev 2007;87:165-217.
[14] Christensen LP. Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. Adv Food Nutr Res 2009;55:1–99.
[15] Choi KT. Botanical characteristics, pharmacological effects and medicinal components of Korean Panax ginseng C A Meyer. Acta Pharmacol Sin 2008;29: 1109-18.
[16] Kim do Y, Yuan HD, Chung IK, Chung SH. Compound K, intestinal metabolite of ginsenoside, attenuates hepatic lipid accumulation via AMPK activation in human hepatoma cells. J Agric Food Chem 2009;57:1532-7.
[17] Han GC, Ko SK, Sung JH, Chung SH. Compound K enhances insulin secretion with beneficial metabolic effects in db/db mice. J Agric Food Chem 2007;55:10641-8.
[18] Choo MK, Sakurai H, Kim DH, Saiki I. A ginseng saponin metabolite suppresses tumor necrosis factor-alpha-promoted metastasis by suppressing nuclear factor-kappaB signaling in murine colon cancer cells. Oncol Rep 2008;19:595-600.
[19] Kang KA, Kim YW, Kim SU, Chae S, Koh YS, Kim HS, et al. G1 phase arrest of the cell cycle by a ginseng metabolite, compound K, in U937 human monocytic leukemia cells. Arch Pharm Res 2005;28:685-90.
[20] Choi K, Choi C. Proapoptotic ginsenosides compound K and Rh enhance Fas-induced cell death of human astrocytoma cells through distinct apoptotic signaling pathways. Cancer Res Treat 2009;41:36-44.
[21] Chae S, Kang KA, Chang WY, Kim MJ, Lee SJ, Lee YS, et al. Effect of compound K, a metabolite of ginseng saponin, combined with gamma-ray radiation in human lung cancer cells in vitro and in vivo. J Agric Food Chem 2009;57: 5777-82.
[22] Noh KH, Son JW, Kim HJ, Oh DK. Ginsenoside compound K production from ginseng root extract by a thermostable beta-glycosidase from *Sulfolobus solfataricus*. Biosci Biotechnol Biochem 2009;73:316-21.
[23] Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J Natl Cancer Inst 1997;89:1881-6.
[24] Lee KW, Kang NJ, Heo YS, Rogozin EA, Pugliese A, Hwang MK, et al. Raf and MEK protein kinases are direct molecular targets for the chemopreventive effect of quercetin, a major flavonol in red wine. Cancer Res 2008;68:946-55.
[25] Hwang JT, Kim YM, Surh YJ, Baik HW, Lee SK, Ha J, et al. Selenium regulates cyclooxygenase-2 and extracellular signal-regulated kinase signaling pathways by activating AMP-activated protein kinase in colon cancer cells. Cancer Res 2006;66:10057-63.
[26] Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, et al. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr Biol 2003;13:2004-8.
[27] Hutchinson DS, Summers RJ, Bengtsson T. Regulation of AMP-activated protein kinase activity by G-protein coupled receptors: potential utility in treatment of diabetes and heart disease. Pharmacol Ther 2008;119:291-310.
[28] Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, et al. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab 2005;2:9–19.
[29] Domotor A, Peidl Z, Vincze A, Hunyady B, Szolcsanyi J, Kereskay L, et al. Immunohistochemical distribution of vanilloid receptor, calcitonin-gene related peptide and substance P in gastrointestinal mucosa of patients with different gastrointestinal disorders. Inflammopharmacology 2005;13:161-77.
[30] Bair AM, Thippegowda PB, Freichel M, Cheng N, Ye RD, Vogel SM, et al. Ca^{2+} entry via TRPC channels is necessary for thrombin-induced NF-kappaB activation in endothelial cells through AMP-activated protein kinase and protein kinase Cdelta. J Biol Chem 2009;284:563-74.
[31] Shibata S. Chemistry and cancer preventing activities of ginseng saponins and some related triterpenoid compounds. J Korean Med Sci 2001;16(Suppl): S28-37.
[32] Wakabayashi C, Murakami K, Hasegawa H, Murata J, Saiki I. An intestinal bacterial metabolite of ginseng protopanaxadiol saponins has the ability to induce apoptosis in tumor cells. Biochem Biophys Res Commun 1998;246:725-30.
[33] Cho SH, Chung KS, Choi JH, Kim DH, Lee KT. Compound K, a metabolite of ginseng saponin, induces apoptosis via caspase-8-dependent pathway in HL-60 human leukemia cells. BMC Cancer 2009;9:449.
[34] Lee SJ, Ko WG, Kim JH, Sung JH, Moon CK, Lee BH. Induction of apoptosis by a novel intestinal metabolite of ginseng saponin via cytochrome c-mediated activation of caspase-3 protease. Biochem Pharmacol 2000;60:677-85.
[35] Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 2005;1:15-25.
[36] Zhang BB, Zhou G, Li C. AMPK: an emerging drug target for diabetes and the metabolic syndrome. Cell Metab 2009;9:407-16.
[37] Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, et al. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. Cancer Res 2007;67:6745-52.
[38] Rattan R, Giri S, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside inhibits cancer cell proliferation in vitro and in vivo via AMP-activated protein kinase. J Biol Chem 2005;280:39582-93.
[39] Huang YC, Lin CY, Huang SF, Lin HC, Chang WL, Chang TC. Effect and mechanism of ginsenosides CK and Rg1 on stimulation of glucose uptake in 3T3-L1 adipocytes. J Agric Food Chem 2010;58:6039-47.
[40] Kim do Y, Park MW, Yuan HD, Lee HJ, Kim SH, Chung SH. Compound K induces apoptosis via CAMK-IV/AMPK pathways in HT-29 colon cancer cells. J Agric Food Chem 2009;57:10573-8.
[41] Collins QF, Liu HY, Pi J, Liu Z, Quon MJ, Cao W. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5′-AMP-activated protein kinase. J Biol Chem 2007;282:30143-9.
[42] Memmott RM, Gills JJ, Hollingshead M, Powers MC, Chen Z, Kemp B, et al. Phosphatidylinositol ether lipid analogues induce AMP-activated protein kinase-dependent death in LKB1-mutant non small cell lung cancer cells. Cancer Res 2008;68:580-8.
[43] Marasa BS, Xiao L, Rao JN, Zou T, Liu L, Wang J, et al. Induced TRPC1 expression increases protein phosphatase 2A sensitizing intestinal epithelial cells to apoptosis through inhibition of NF-kappaB activation. Am J Physiol Cell Physiol 2008;294:C1277-87.
[44] Venkatachalam K, van Rossum DB, Patterson RL, Ma HT, Gill DL. The cellular and molecular basis of store-operated calcium entry. Nat Cell Biol 2002;4: E263-72.
[45] Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 2003;4:552-65.