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

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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.
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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-

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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).

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