Abstract. Previously, we reported that 20-O-(β-D-glucopyranosyl)-20(S)-protopanaxadiol (Compound K, a metabolite of ginseng saponin) induces mitochondria-dependent and caspase-dependent apoptosis in HT-29 human colon cancer cells via the generation of reactive oxygen species. The aim of the present study was to elucidate the mechanism underlying apoptosis induced by Compound K with respect to endoplasmic reticulum (ER) stress in HT-29 cells. In the present study, Compound K induced apoptotic cell death as confirmed by DNA fragmentation and apoptotic sub-G₁ cell population. Compound K also induced ER stress as indicated by staining with ER tracker, cytosolic and mitochondrial Ca²⁺ over-loading, phosphorylation of protein-kinase-like endoplasmic reticulum kinase (PERK), phosphorylation of eukaryotic initiation factor-2α (eIF-2α), phosphorylation of IRE-1, splicing of ER stress-specific X-box transcription factor-1 (XBP-1), cleavage of activating transcription factor-6 (ATF-6), upregulation of glucose-regulated protein-78 (GRP-78/BiP) and CCAAT/enhancer-binding protein-homologous protein (CHOP), and cleavage of caspase-12. Furthermore, downregulation of CHOP expression using siCHOP RNA attenuated Compound K-induced apoptosis. Taken together, these results support the important role of ER stress response in mediating Compound K-induced apoptosis in human colon cancer cells.

Introduction

The endoplasmic reticulum (ER) is a secretory organelle, which plays a critical role in lipid synthesis and protein folding and modification before export to the Golgi body. Protein folding is impaired under unbalanced redox state, failure of protein synthesis, misfolding, transport or degradation and Ca²⁺ over-loading, which can result in ER stress (1-3). ER stress leads to the accumulation of unfolded proteins in the ER lumen where they form highly toxic aggregates. If these toxic aggregates reach critical levels, ER stress response and apoptotic signaling are initiated (4).

The ER stress response is primarily regulated by three ER transmembrane proteins; protein kinase-like endoplasmic reticulum kinase (PERK), phosphorylation of eukaryotic initiation factor-2α (eIF-2α), phosphorylation of IRE-1, splicing of ER stress-specific X-box transcription factor-1 (XBP-1), cleavage of activating transcription factor-6 (ATF-6), upregulation of glucose-regulated protein-78 (GRP-78/BiP) and CCAAT/enhancer-binding protein-homologous protein (CHOP), and cleavage of caspase-12. Furthermore, downregulation of CHOP expression using siCHOP RNA attenuated Compound K-induced apoptosis. Taken together, these results support the important role of ER stress response in mediating Compound K-induced apoptosis in human colon cancer cells.

Correspondence to: Professor Jin Won Hyun or Professor Weon Young Chang, School of Medicine, Jeju National University, 66 Jejudaehakno, Jeju 690-756, Republic of Korea
E-mail: jinwonh@jejunu.ac.kr
E-mail: orkorea@jejunu.ac.kr

Key words: Compound K, apoptosis, endoplasmic reticulum stress, CCAAT/enhancer-binding protein-homologous protein
apoptotic signaling and increases CHOP expression. CHOP is the major component of the ER stress pathway, and CHOP knockdown cells are resistant to ER stress-induced apoptosis (11). Caspase-12 has also been implicated in ER stress-mediated cell death, and caspase-12 knockdown mice are resistant to ER stress-induced apoptosis (12).

Accumulating evidence suggests that anticancer agents are toxic to cancer cells because they increase oxidative stress, pushing the cancer cells beyond their limits (13-15). Cytotoxic reactive oxygen species (ROS) appears to be triggered by activation of the mitochondrial-dependent cell death pathway via Bcl-2 family proteins, with consequent mitochondrial membrane permeabilization and apoptosis (16).

20-O-(β-D-glucopyranosyl)-20(R)-protopanaxadiol (Compound K, Fig. 1A) is the main metabolite of protopanaxadiol-type ginsenosides formed in the intestine after oral administration (17-20). Ginseng saponin was reported to show beneficial roles on abnormal coronary contraction (21) and possess therapeutic effect on skin wound healing (22). Our previous studies show that Compound K enhances gamma ray-induced apoptosis via generation of ROS and disruption of the mitochondrial membrane in human lung cancer cells (23), and induces mitochondria- and caspase-dependent apoptosis via the generation of ROS in human colon cancer cells (24). Furthermore, Compound K exhibits anti-proliferative effects against cancer cells, which are mediated via apoptosis (23-29). Despite evidence for the anti-proliferative effects of Compound K, the underlying cytotoxic mechanisms with respect to ER stress-mediated apoptosis have not been reported. Therefore, the aim of the present study was to determine the role of ER stress in mediating Compound K-induced apoptosis in HT-29 human colon cancer cells.

Materials and methods

Preparation of Compound K. Compound K was provided by Professor Dong Hyun Kim (Kyung Hee University, Seoul, Republic of Korea). Compound K was prepared by the incuba- tion 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/H₂O (65:35:10). The isolated Compound K was characterized by mass spectroscopy and ¹H and ¹³C nuclear magnetic resonance (NMR) spectrometry.

Reagents. Propidium iodide (PI) was purchased from Sigma Chemical Company (St. Louis, MO, USA). CHOP, caspase-12, ATF-6 and β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Phospho PERK, phospho eIF-2α, phospho IRE-1, XBP-1 and GRP-78 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were of analytical grade.

Cell culture. HT-29 human colon cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained at 37˚C in an incubator with a humidified atmosphere of 5% CO₂ in air. Cells were cultured in RPMI-1640 containing 10% heat-inactivated fetal calf serum, streptomycin (100 µg/ml) and penicillin (100 U/ml).

DNA fragmentation. Cells were seeded at a concentration of 5x10⁴ cells/ml, and 16 h after plating, were treated with Compound K at 20 µg/ml and incubated for 48 h. Cellular DNA fragmentation was assessed using the cytoplasmic histone-associated DNA fragmentation kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer’s instructions.

Detection of sub-G₁ hypodiploid cells. The amount of apoptotic sub-G₁ hypodiploid cells was determined by flow cytometry (30). Cells were seeded at a six-well plate at a concentration of 1x10⁵ cells/ml, and 16 h after plating, were treated with Compound K at 20 µg/ml for 48 h. Harvested cells were washed twice with phosphate buffered saline (PBS) and fixed in 70% ethanol for 30 min at 4˚C. Cells were incubated for 30 min in the dark at 37˚C in 1 ml PBS containing 100 µg PI and 100 µg RNase A. Flow cytometric analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). The proportion of sub-G₁ hypodiploid cells was assessed by the histograms generated using the computer program Cell Quest and Mod-Fit (Becton Dickinson).

Cytosolic Ca²⁺ measurement. Cytosolic Ca²⁺ was detected with the fluorescent probe Fluo4 AM. Cells were seeded at a concentration of 1x10⁵ cells/ml, and 16 h after plating, were treated with Compound K at 20 µg/ml and incubated for 24 h. Cells were then harvested and loaded with 10 µM of Fluo4 AM for 30 min at 37˚C and the supernatant was removed by suction and after trypsin treatment, cells were washed with PBS. The fluorescence of Fluo4 AM-loaded cells was measured using a flow cytometer.

Measurement of mitochondrial Ca²⁺. A Rhod2 AM probe (Molecular Probes, Eugene, OR, USA) was used to determine mitochondrial Ca²⁺ level (31). Rhod2 AM has a net positive charge, which facilitates its sequestration into mitochondria via membrane potential-driven uptake. The use of Rhod2 AM enhances the selectivity for mitochondrial loading because the dye exhibits Ca²⁺-dependent fluorescence only after it is oxidized, and this occurs preferentially within mitochondria. Cells were seeded at a concentration of 1x10⁵ cells/ml, and 16 h after plating, were treated with Compound K at 20 µg/ml and incubated for 24 h. Cells were harvested, washed, and resuspended in PBS containing Rhod2 AM (1 µM). After 15 min of incubation at 37˚C, cells were washed, suspended in PBS and analyzed by flow cytometry. For image analysis, cells were loaded with Rhod2 AM and incubated for 30 min at 37˚C. Cells were then washed, and the stained cells mounted onto microscope slides with mounting medium (Dako, Carpinteria, CA, USA). Microscopic images were examined using a confocal laser scanning microscope and the 5 PASCAL program (Carl Zeiss, Jena, Germany).

Fluorescent microscopy and ER staining. Cells were seeded in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, USA) at a density of 1x10⁵ cells/ml, and 16 h after plating, were treated with Compound K at 20 µg/ml and incubated for
For ER staining, the ER-Tracker Blue-White DPX probe (Molecular Probes) was added to the cells and incubated for 30 min under the same growth conditions. The loading solution was removed and cells were then washed with PBS before adding fresh medium without the stain. Microscopic images were collected using the laser scanning microscope 5 Pascal program (Carl Zeiss) as described above.

**Western blot analysis.** Cells were seeded at a concentration of 1.5x10⁵ cells/ml, and 16 h after plating, were treated with Compound K at 20 µg/ml. After 3, 6, 12, 24 or 48 h, cells were harvested, washed twice with PBS, lysed on ice for 30 min in 100 µl of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% NP40] and then centrifuged at 13,000 x g for 15 min. The supernatants were collected from the lysates and the protein concentrations were determined. Aliquots of the lysates (40 µg of protein) were boiled for 5 min and electrophoresed in 10% SDS-PAGE gel. The proteins in the gels were transferred onto nitrocellulose membranes and incubated with the primary antibodies. The membranes were subsequently incubated with the secondary immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK), and then exposed to X-ray film.

**Transient transfection of small RNA interference (siRNA).** Cells were seeded in 24-well plate at a density of 1.5x10⁵ cells/ml and allowed to reach ~50% confluence on the day of transfection. The siRNA construct used were: mismatched siControl RNA (Santa Cruz Biotechnology) and siCHOP RNA (Bioneer Corporation, Bioneer, South Korea). Cells were transfected with 10-50 nM siRNA using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h, cells were treated with Compound K for 48 h and examined by western blot analysis, DNA fragmentation assay and PI staining.

**Statistical analysis.** All the measurements were made in triplicate and all values are represented as the mean ± standard error of the mean (SEM). Differences in results were analyzed using analysis of variance (ANOVA) and the Tukey test. p<0.05 was considered significant.

**Results**

**Induction of apoptosis in human colon cancer cells by Compound K treatment.** A recent study showed that Compound K induces mitochondria- and caspase-dependent apoptosis in HT-29 cells via the generation of ROS (24). The results of the present study showed that 20 µg/ml of Compound K, which is a concentration of 50% growth inhibition, increased DNA fragmentation and sub-G₁ phase of cell population, which are hallmarks of apoptosis (Fig. 1B and C).

**Compound K induces cytosolic and mitochondrial Ca²⁺ overloading and ER stress.** Depletion of ER calcium stores induces ER stress, which leads to an increase in cytosolic and mitochondrial Ca²⁺ levels (32,33). Therefore, the effect of Compound K on the mobilization of Ca²⁺ was examined. Compound K resulted in significant increases in cytosolic (Fig. 2A) and mitochondrial Ca²⁺ levels (Fig. 2B and C) at 24 h after Compound K treatment. ER stress increases the fluorescence intensity of the ER-Tracker Blue-White DPX dye (34). As shown in Fig. 2D, Compound K significantly increased the staining intensity of this dye, suggesting the induction of ER stress.

**Compound K increases the level of ER stress-related proteins.** During ER stress, the activated phospho form of PERK phosphorylates eIF-2α, leading to the attenuation of translational initiation and protein synthesis (35,36). As shown in Fig. 3, Compound K induced the expression of both phosphorylated PERK and phosphorylated eIF-2α in a time-dependent manner. Also, the activated phospho form of IRE-1 splices XBP-1, leading to increase in membrane phospholipids and expansion of the surface area and volume of the rough ER (37). As shown in
Fig. 3, Compound K induced the expression of phosphorylated IRE-1 and spliced XBP-1 protein in a time-dependent manner. Other hallmarks of the ER stress responses include activation of ATF-6, the subsequent induction of GRP-78 and CHOP, and the activation of caspase-12 (32,38,39). Compound K also enhanced ATF-6 activation, induced the expression of GRP-78 and CHOP, and activated caspase-12 (Fig. 3).

Suppression of CHOP expression attenuates Compound K-induced apoptosis. CHOP plays a proapoptotic role during ER stress (38,40). Suppression of siRNA-mediated CHOP expression attenuated the apoptotic cell death induced by Compound K, which was confirmed by the DNA fragmentation pattern and the sub-G1 cell population (Fig. 4A and B). These results suggest that upregulation of CHOP may, in part, be involved in Compound K-induced apoptosis.

Discussion

Although Compound K induces apoptosis in many cancer cell lines (23-29), the underlying mechanisms are not well understood. The central novel finding of this study provides important evidence to support the involvement of ER stress in the induction of apoptosis by Compound K in HT-29 cells. The ER is the primary site of protein synthesis, folding and trafficking. Under various stressful conditions, the accumulation
of unfolded or misfolded proteins in the ER results in ER stress (1,2). Under conditions of ER stress, the elevation of cytosolic or mitochondrial Ca\(^{2+}\) levels, or the depletion of ER Ca\(^{2+}\) stores are typical ER stress responses of cells. The present study shows that Compound K induced the elevation of cytosolic and mitochondrial Ca\(^{2+}\) levels and ER staining. Compound K also induced apoptosis as assessed by increased DNA fragmentation and sub-\(G_1\) phase of cell population. The experimental evidence presented here shows that the induction of ER stress-related proteins may be involved in Compound K-induced apoptosis as: i) Compound K induces phosphorylation of PERK and eIF-2\(\alpha\); ii) Compound K induces phosphorylation of IRE-1 and the spliced XBP transcription factor; iii) Compound K induces cleavage of ATF-6, and subsequent GRP-78 and CHOP expression; and iv) Compound K induces caspase-12 cleavage. Among ER-associated apoptotic molecules, CHOP and caspase-12 are major proapoptotic factors that are closely associated with ER stress (11). Taken together, these observations demonstrate that Compound K induces ER-mediated apoptosis. ER stress response pathways are normally activated as a protective mechanism to ensure cell survival (41). However, during severe ER stress, activation of these pathways leads to increased CHOP expression, which is a crucial element that switches ER stress signaling from pro-survival to proapoptosis (42). The CHOP protein is a member of the CCAAT/enhancer-binding proteins and functions as a dominant-negative inhibitor of gene transcription (38). Expression of CHOP is mainly regulated at the transcriptional level through the PERK/eIF-2\(\alpha\)/ATF-6 pathway (38). CHOP knockout mice show reduced apoptosis in response to ER stress (43). Therefore, CHOP is one of the components of the ER stress-mediated apoptosis pathway. In the present study, suppression of CHOP using CHOP siRNA attenuated Compound K-induced apoptosis.

In summary, Compound K induces the apoptosis of HT-29 colon cancer cells which is mediated by ER stress signaling pathway and this is the first report to reveal that the association between the capacity of Compound K to induce ER stress and apoptosis in colon cancer cells.

**Acknowledgements**

This work was supported by the research grant from Jeju National University Hospital. And this study was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health and Welfare, Republic of Korea (1120340).
References


16. ZHANG et al: ENDOPLASMIC RETICULUM STRESS BY COMPOUND K