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Title: Ginsenoside Rg3 disrupts actin-cytoskeletal integrity leading to contractile dysfunction and apoptotic cell death in vascular smooth muscle

Article Type: Full Length Article

Keywords: ginseng; ginsenoside Rg3; actin cytoskeleton; vascular toxicity; apoptosis; actin-targeting anti-cancer agents

Abstract: Ginseng and its major ingredients, ginsenosides are widely consumed for various health problems without a special precaution. Previously, we demonstrated that Rg3, one of ginsenosides, may cause contractile dysfunction, and structural damage on vascular smooth muscle through apoptotic cell death. However, the mechanism underlying Rg3-induced vascular toxicity was not fully elucidated. Here we demonstrated that the vascular toxicity of Rg3 occurs through actin disruption and Bmf-initiated mitochondrial apoptotic pathway, the mechanism which may contribute to its anti-cancer effects. In rat vascular smooth muscle cells, Rg3 induced apoptosis and contractile dysfunction as determined by caspase-3 activation, TUNEL staining, and insufficient myosin light chain phosphorylation. Rg3 induced actin degradation with Rho A inactivation, which was followed by mitochondrial translocation of Bmf and dissociation of mitochondrial membrane potential. Pre-treatment of jasplakinolide, an inhibitor of actin depolymerization, significantly blocked Rg3-induced apoptosis. Notably, Rg3 induced actin disruption in hepatoma cell line, HepG2, which could be reversed by jasplakinolide, reflecting that the same mechanism may contribute to the anti-cancer effects of Rg3 at least in part. Collectively, these results demonstrated that Rg3 may induce vascular toxicity as well as anti-cancer effects through disrupting actin, which may alert the potential toxicity associated with inadvertent use of ginseng

Response to Reviewers: Please see the attached file

Responses to Reviewer #1:

Q1. Why the authors consider the vascular toxicity of ginseng a problem when the anticancer activity of a given compound (e.g., ginseng) could be related to its vascular toxicity, especially when considering the involvements of vascular cells in the metastatic phenomena. Authors should try to better explain potential correlation and differences on the vascular toxicity and anti-cancer effects of the tested compound.

A1. It is a good point to mention that vascular toxicity against newly formed blood vessels around the cancer tissues through angiogenesis may be helpful in managing cancer tissues. Actually Thoenes et al. (2008) suggests that the actin-disruption in newly formed vascular endothelial cells may be helpful in suppressing angiogenesis.

We wanted to underscore in our study is the vascular toxicity of ginsenoside Rg3 against the vascular muscle tissues of existing normal blood vessels. While the vascular toxicity of Rg3 against the newly formed vessels around cancer tissues of cancer patients may be beneficial, it may be harmful towards people without cancer tissues such as those who consume ginsenosides for cancer prevention. We reflected this point as follows in the revision.

“Actin-disruption effects may also be beneficial for the treatment of cancer through impairing angiogenesis through suppressing endothelial proliferation (Thoenes et al., 2008).”

Q2. Many of the reported side effects due to ginseng intake do not appear to involve vascular cells. Why the author perform the experiments on Vascular Smooth Muscle Cells?

A2. As described in the introduction, ginseng is widely used for improvement of cardiovascular function and several studies have shown that consumption of ginseng could be related to vascular toxicity revealed as hypertension, hypotension and vascular dysfunction.

“Especially, ginseng products are widely consumed for the improvement of cardiovascular function (Stavro et al., 2006) but the potential vascular toxicity of ginseng and ginsenosides is drawing attention with respect to hypertension, hypotension and vascular dysfunction (Ernst, 2002; Lee et al., 2010; Siegel, 1980).” in the introduction.

Moreover, we have previously reported that ginsenoside Rg3 could cause vascular smooth muscle dysfunction leading to apoptosis and structural remodeling of blood vessel (Lee et al., 2010).

Against this backdrop, we addressed the effects of Rg3 in vascular smooth muscle cells in the present study. We hope that the reviewer could agree with our view.

Q3. In their manuscript the authors stated " However, known molecular mechanisms for Rg3 action, like ROS generation, NF-κB, ERK activation (Choi et al., 2013; Kim et al., 2014), and AMPK-mediated mitochondrial apoptotic pathways (Yuan et al., 2010) cannot fully explain this distinct pattern of functional and structural vascular toxicity". I am not sure to understand what does "distinct pattern of functional and structural vascular toxicity" mean.

A3. Thank you for a kind comment. We could also find that the paragraph needs clarification. We could find little association between functional vascular toxicity of Rg3 and the conventionally known molecular mechanisms of anti-cancer effects of Rg3 like ROS generation, NF-κB, ERK activation and AMPK-mediated mitochondrial apoptotic pathways. We searched for other anti-cancer mechanism which can be shared with vascular toxicity. Through the literature search, we found that actin-targeting anti-cancer agents (ATACs) have vascular toxicity problems. On this context, we investigated the vascular toxicity of Rg3 with respect to actin integrity. We revised the paragraph as follows,

“Recently, we demonstrated that Rg3, a ginsenoside with diverse potent biological activities including the anti-hypertensive (Kim et al., 1999; Kim et al., 2003), neuroprotective (Tian et al., 2005), anti-cancer (Sun et al., 2017), anti-metastatic (Choi et al., 2013) and anti-proliferative activities (He et al., 2011), can inflict irreversible damages on the contractile function of the vascular smooth muscle and eventually lead to the apoptosis of smooth muscle cells and vascular remodeling in vivo (Lee et al., 2010). Especially, Rg3 is known to have anti-metastatic and anti-proliferative effects (Christensen, 2009; Helms, 2004). It is widely consumed as dietary supplements for various health benefits including the prevention of cancers (Guangzhou Endless Biotech, 2017; Supplement Healthstore, 2017) and has even examined in clinical trials for anti-cancer effects as well (Li et al., 2016; Lu et al., 2008; Zhou et al., 2016). We hypothesized that the vascular toxicity of Rg3 could be linked to its anti-cancer activities since anti-proliferative and anti-metastatic effects may appear as toxicity against normal tissues. However, conventionally known molecular mechanisms for the anti-cancer activities of Rg3, like ROS generation, NF-κB, ERK activation (Choi et al., 2013; Kim et al., 2014), and AMPK-mediated mitochondrial apoptotic pathways (Yuan et al., 2010) could not fully explain the vascular toxicity of Rg3 with respect to contractile dysfunction.”

Q4. All the paragraph starting with "Incidentally, anti-metastatic and anti-proliferative effects of ginsenoside" appears to completely dissociate from the rest of the introduction. A better introduction for this cancer-related effect should be fused

Moreover, the starting from "incidentally" and going forward in the manuscript, the introduction focuses on the mechanistic aspects of ATAC drugs, and in this regards it is not clearly explained the importance of such aspect about ginseng and its cardiotoxicity.

A4. In line with the revision of the paragraph before the one, we removed the sentence starting from “incidentally” and revised it as follows,

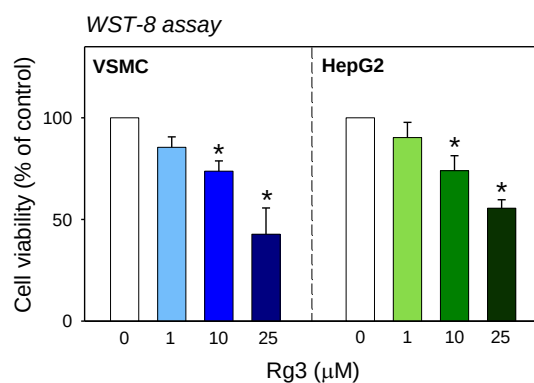
“Recently, a new class of anti-cancer agents with a novel anti-proliferative mechanism has been introduced, which targets the actin-cytoskeleton of cancer cells (Bonello et al., 2009). These actin-targeting anti-cancer agents (ATACs) attack the actin cytoskeleton, which triggers the disintegration of actin, and the subsequent dissociation and mitochondrial translocation of actin-associated Bmf, a pro-apoptotic BH-3 only Bcl family protein. Pro-apoptotic Bmf, then initiates the apoptotic process in the mitochondria of the affected cancer cells (Desouza et al., 2012; Puthalakath et al., 2001). ATACs have safety issues however, since the actin cytoskeleton of cancer cells could not be differentiated from that of normal cells (Stehn et al., 2013), which plays a pivotal role in the function or survival of cells (Franklin-Tong and Gourlay, 2008; Gourlay and Ayscough, 2005; Yamazaki et al., 2005).”

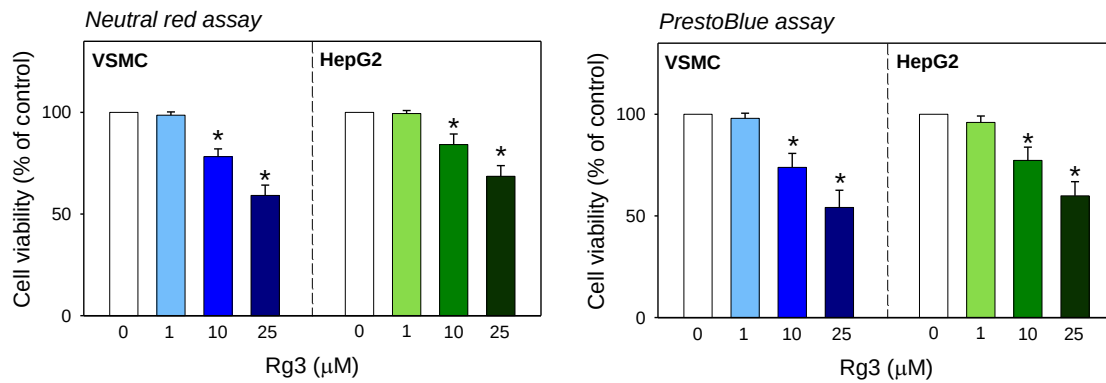
Q5. Considering the introduction the manuscript and the experiments performed, I suggest using the term vascular toxicity rather than cardiotoxicity.

A5. We see that the reviewer is mentioning cardiovascular toxicity. As advised, we replaced cardiovascular toxicity by vascular toxicity throughout the whole manuscript.

Q6. Tetrazolium-based compound (e.g., MTT, WST) appears to interact with natural compound providing artifacts. I suggest to used, at least, one more additional method to determinate the cell proliferation

A6. Thank you for your comment. As the reviewer suggested we tested the effects of ginsenoside Rg3 in cell viability (cell proliferation) with other methods, PrestoBlue and neutral red uptake assay. As seen below both PrestoBlue and neutral red uptake assay showed similar inhibitory effects on cytotoxicity compared with previous WST-8 assay. As ginsenoside Rg3 also showed similar cytotoxic effects in other cell viability assay, we would like to present only the WST-8 assay which is commonly used. We hope that the reviewer will be satisfied with our response.





Q6. Are the used concentrations reflective of potential *in vivo* effects! In some assays, the concentration that provides significant results is 25 micromolar, which is quite far from 6 micromolar, the concentration *in vivo* in the blood

A6. As you see in the results, statistically significant effects were observed from the concentrations of 10 μM which is close to the concentration *in vivo* in the blood, 6 μM. We included the concentration (10~25 μM) that showed significant effects in the part of discussion for clear understanding.

Responses to Reviewer #2:

Q1. The materials and methods section does not allow to reproduce your experimental approach based on a poor description.

E.g., how have the smooth muscle cells been obtained? What was the concentration of the collagenase and elastase? At what density have the cells been seeded?

I do not believe that the cells have been cultured in an atmosphere consisting of 95% O₂ as described in your manuscript.

Please check very carefully the materials and methods section and provide a methodological description allowing researchers to reproduce your approach.

A1. We checked carefully the materials and methods section and revised it. Briefly we added more details in isolation of smooth muscle cells, especially the concentration of collagenase and elastase. And we provided the information of cell number seeded in each experiments. Also corrected the cell culture condition into 95% air/5% CO₂. We appreciate your careful check of our manuscript

Ginsenoside Rg3 disrupts actin-cytoskeletal integrity leading to contractile dysfunction and apoptotic cell death in vascular smooth muscle

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Competing Financial Interests Declaration: None

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Abstract

Ginseng and its major active ingredients, ginsenosides are widely consumed for various health benefits including the prevention or treatment of cancer without any special precaution. Previously, we demonstrated that Rg3, one of ginsenosides with a potent anti-cancer activity, may cause contractile dysfunction, and structural damage against normal vascular smooth muscle through apoptotic cell death. However, mechanism underlying the vascular toxicity of Rg3 was not fully elucidated. Here we demonstrated that the vascular toxicity of Rg3 occurs through actin disruption and Bmf-initiated mitochondrial apoptotic pathway, the mechanism which may be shared with its anti-cancer effects. In normal rat primary vascular smooth muscle cells, Rg3 induced apoptosis and contractile dysfunction as determined by caspase-3 activation, TUNEL staining, and insufficient myosin light chain phosphorylation. Rg3 induced actin degradation with RhoA inactivation, followed by mitochondrial translocation of Bmf and dissociation of mitochondrial membrane potential (ϕ). Pre-treatment of jasplakinolide, an inhibitor of actin depolymerization, significantly blocked Rg3-induced ϕ dissipation and apoptosis. Notably, Rg3 also induced actin disruption in hepatoma cell line, HepG2, which was reversed by jasplakinolide, confirming that the anti-cancer effects of Rg3 shares the mechanism at least in part. Collectively, these results demonstrated that Rg3 may induce vascular toxicity as well as anti-cancer effects through disrupting actin, suggesting the potential toxicity associated with inadvertent use of ginseng and its products.

Key words: ginseng; ginsenoside Rg3; actin cytoskeleton; vascular toxicity; apoptosis; actin-targeting anti-cancer agents

1. Introduction

Panax ginseng C. A. meyer, ginseng is a symbolic herbal medicine heavily consumed in Eastern Asian countries that include Korea, China and even US as dietary supplement, tonics, alternative medicines and even as panacea (Baeg *et al.*, 2013). Diverse biological activities have been reported for ginseng extract and its ingredients, which include anti-aging, anti-cancer, anti-hypertensive, anti-inflammatory, and anti-oxidant activities (Karmazyn *et al.*, 2011). These biological activities of ginseng are mostly attributable to ginsenosides, a major saponic component enriched in it. Along with the potent therapeutic effects, a concern over the potential toxicity of ginseng and ginsenosides has been raised. It is reported that long-term consumption of ginseng at high doses is not without toxicities (Paik *et al.*, 2015). Siegel conducted two year studies in the subjects consuming ginseng extracts, which revealed frequent side effects like morning diarrhea, skin eruption, nervousness, sleeplessness, hypertension, edema, decreased appetite, depression, and hypotension (Siegel, 1979). Especially, ginseng products are widely consumed for the improvement of cardiovascular function (Stavro *et al.*, 2006) but the potential **vascular toxicity** of ginseng and ginsenosides is drawing attention with respect to hypertension, hypotension and vascular dysfunction (Ernst, 2002; Lee *et al.*, 2010a; Siegel, 1980).

Despite many lines of experimental and clinical evidence for the **vascular toxicity** of ginseng and ginsenosides, detailed mechanistic explanation has been rarely provided. Recently, we demonstrated that Rg3, a ginsenoside with diverse potent biological activities including the anti-hypertensive (Kim *et al.*, 1999; Kim *et al.*, 2003), neuroprotective (Tian *et al.*, 2005), anti-cancer (Sun *et al.*, 2017), anti-metastatic (Choi *et al.*, 2013) and anti-proliferative activities (He *et al.*, 2011), can inflict irreversible damages on the contractile

function of the vascular smooth muscle and eventually lead to the apoptosis of smooth muscle cells and vascular remodeling *in vivo* (Lee, et al., 2010a). Especially, Rg3 is known to have anti-metastatic and anti-proliferative effects (Christensen, 2009; Helms, 2004). It is widely consumed as dietary supplements for various health benefits including the prevention of cancers (GuangzhouEndlessBiotech, 2017; SupplementHealthstore, 2017) and has even examined in clinical trials for anti-cancer effects as well (Li *et al.*, 2016; Lu *et al.*, 2008; Zhou *et al.*, 2016). We hypothesized that the vascular toxicity of Rg3 could be linked to its anti-cancer activities since anti-proliferative and anti-metastatic effects may appear as toxicity against normal tissues. However, conventionally known molecular mechanisms for the anti-cancer activities of Rg3, like ROS generation, NF- κ B, ERK activation (Choi, *et al.*, 2013; Kim *et al.*, 2014), and AMPK-mediated mitochondrial apoptotic pathways (Yuan *et al.*, 2010) could not fully explain the vascular toxicity of Rg3 with respect to contractile dysfunction.

Recently, a new class of anti-cancer agents with a novel anti-proliferative mechanism has been introduced, which targets the actin-cytoskeleton of cancer cells (Bonello *et al.*, 2009). These actin-targeting anti-cancer agents (ATACs) attack the actin cytoskeleton, which triggers the disintegration of actin, and the subsequent dissociation and mitochondrial translocation of actin-associated Bmf, a pro-apoptotic BH-3 only Bcl family protein. Pro-apoptotic Bmf, then initiates the apoptotic process in the mitochondria of the affected cancer cells (Desouza *et al.*, 2012; Puthalakath *et al.*, 2001). ATACs have safety issues however, since the actin cytoskeleton of cancer cells could not be differentiated from that of normal cells (Stehn *et al.*, 2013), which plays a pivotal role in the function or survival of cells (Franklin-Tong *et al.*, 2008; Gourlay *et al.*, 2005; Yamazaki *et al.*, 2005).

Especially for the muscle tissues where actin is critical for the generation of contractile forces (Reho *et al.*, 2014), ATACs may be highly toxic, of which point is well corroborated by the reports of their adverse effects against heart, vascular smooth muscle and diaphragm (Stehn *et al.*, 2006). Cytochalasin, a representative ATAC which impairs actin polymerization and thereby, disintegrates actin structure, suppresses the proliferation and invasion of cancer cells (Trendowski, 2015), is well known to induce cardiac and vascular toxicity through causing contractile dysfunction via inhibiting MLC phosphorylation (Bruijns *et al.*, 2001).

The anti-cancer mechanism of ATACs gave a clue to us for the explanation of the vascular toxicity of Rg3 since the impairment of actin cytoskeleton can induce both contractile dysfunction and apoptotic cell death. Here, we examined whether Rg3 can induce toxicity against vascular smooth muscle in the aspect of actin-disruption and subsequent activation of Bmf-initiated apoptosis. In addition, we also investigated whether Rg3 could affect the actin cytoskeleton of cancer cell-line to explore if the anti-cancer mechanism of Rg3 converges with that of vascular toxicity.

2. Materials & Methods

2.1. Reagents.

The following chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO): ginsenoside Rg3, serotonin hydrochloride (5-HT) and dimethyl sulfoxide (DMSO). Jaspilakinolide was purchased from Molecular Probes Inc. (Eugene, OR) and reagents and media used in cell culture were purchased from Gibco Co. (Carlsbad, CA). All other reagents used were of the highest purity available.

2.2. Animals.

Male Sprague-Dawley (SD) rats (SamTako, Korea) weighing 150-170 g were used in experiment for primary culture of vascular smooth muscle cells (VSMCs). Before the experiments, the animals were acclimated for 1 week under controlled laboratory conditions of temperature at $22 \pm 2^{\circ}\text{C}$ and humidity at $55 \pm 5\%$ with 12 hr light/12 hr dark cycle. Normal diet (Cargill Agri Purina, Inc., Korea) and water were provided *ad libitum*. All protocols were approved by the Ethics Committee of the Animal Service Center at Seoul National University.

2.3. Cell culture.

After thoracic aorta was isolated from SD rats, the endothelium was removed by gently rubbing the intimal surface, and adventitia was removed using collagenase (150 U/mL, Worthington Biochemical Co., Lakewood, NJ). The thoracic aorta was chopped finely and vascular smooth muscle cells (VSMCs) were liberated from the tissue using collagenase (150 U/mL) and elastase (1.5 U/mL, Worthington Biochemical Co.). VSMCs were incubated in

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in a 95% air/5% CO₂ and 37°C incubator. VSMCs at passage 1-3 were used for experiments. The human HepG2 cell line was obtained from American Type Culture Collection (Manassas, VA). HepG2 cells were incubated in low glucose DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in a 95% air/5% CO₂ and 37°C incubator. Medium was exchanged every 2-3 days.

2.4. Terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining of VSMCs and HepG2 cells.

For TUNEL staining in VSMCs and HepG2 cells, cells (2x10⁵ cells) were grown on collagen coated coverslips in 6-well plates, incubated with ginsenoside Rg3 with or without jasplakinolide 50 nM and then TUNEL stained using Click-iT[®] TUNEL Alexa Fluor[®] Imaging Kit (Molecular Probes Inc., Eugene, OR), according to the manufacturer's instruction. In brief, cells were fixed in 4% formaldehyde in phosphate buffered saline (PBS; 1 mM KH₂PO₄, 154 mM NaCl, 3 mM Na₂HPO₄, pH 7.4) for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature and then incubated with TUNEL reaction mixture containing TdT in a humidified chamber for 60 min at 37°C. After TUNEL reaction, Click-iT reaction mixture containing Alexa 488 azide for 30 min at room temperature, protected from light exposure. Hoechst 33342 was counterstained to examine nuclear morphology. After staining, fluorescent images were obtained using Axiovert 200M fluorescence microscope (Carl Zeiss, Jena, Germany). Intensity of fluorescence was analyzed by ImageJ program (NIH, Bethesda, MD).

2.5. Determination of RhoA activation.

RhoA activity was measured using a commercially available Rho/Rac-1/Cdc42 activation assay kit (Cytoskeleton Inc., Denver, CO) as instructed by the manufacturer. Primary cultured rat VSMCs grown in T-25 flasks (80-90% confluent) were incubated with ginsenoside Rg3 (0, 1, 10, 25 μ M) for 1 hr and then, stimulated with 5-HT 10^{-5} M for 10 min. The cells were harvested in lysis buffer containing protease inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL) on ice. Cell debris was removed by a 15 min centrifugation at 9,800 g, and the supernatant was incubated with rhotekin-RBD beads specifically recognizing active RhoA for 1 hr at 4°C. Beads were washed, and the precipitated active RhoA were detected by western blotting and incubation with specific monoclonal antibody against RhoA (#ARH03, Cytoskeleton Inc., Denver, CO) overnight at 4°C.

2.6. Visualization of actin filament in VSMCs and HepG2 cells.

VSMCs and HepG2 cells (2×10^5 cells) grown on collagen coated coverslip in 6-well plates were incubated with ginsenoside Rg3 (0, 1, 10, 25 μ M) for 4 hr. Cells were fixed with 4% formaldehyde in PBS for 10 min, followed by permeabilization with 0.5% Triton X-100 for 5 min and blocking with 2% bovine serum albumin (BSA) for 1 hr at room temperature. To visualize the actin filament, rhodamine conjugated phalloidin (F-actin) and Alexa 488 conjugated DNase I (G-actin) (Molecular Probes Inc.) was incubated for 1 hr at room temperature, protected from light exposure. Fluorescence images were obtained using Carl Zeiss Axiovert 200M and analyzed by ImageJ program.

2.7. Mitochondrial fractionation and analysis of Bmf translocation.

After cultured rat VSMCs and HepG2 cells grown in 100 mm dish (80-90% confluent) were incubated with ginsenoside Rg3 25 μ M with or without jasplakinolide 50 nM for 4 hr, cytosolic and mitochondrial fractions of VSMCs and HepG2 cells were isolated using a mitochondria isolation kit (Thermo Fisher Scientific Inc., Rockford, IL) according to manufacturer's instruction. Subcellular protein extracts were suspended in Laemmli sample buffer (Bio-Rad, Hercules, CA) and 10 μ g protein of cytosolic fraction and 4 μ g of membrane fraction were subjected to western blot analysis. Blots were detected by incubation with primary antibody against Bmf (#5889, Cell Signaling Technology Inc., Danvers, MA) overnight at 4°C. Antibodies against α -tubulin (#2125) and cytochrome c oxidase (COX) IV (#4844) (Cell Signaling Technology Inc.) were used as loading controls for cytosolic and mitochondrial proteins, respectively.

2.8. Determination of mitochondrial membrane potential.

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were determined with the cationic fluorochrome JC-1 (Sigma-Aldrich Co.), which accumulates in mitochondria as red fluorescent aggregates at intact $\Delta\Psi_m$, whereas exists in green fluorescent monomeric form at decreased $\Delta\Psi_m$. VSMCs (2×10^5 cells) were grown in 6-well plates with collagen coated coverslip and treated with ginsenoside Rg3 with or without jasplakinolide 50 nM for 24 hr. VSMCs were incubated with JC-1 (final 2 μ g/ml) for 15 min at 37°C and fluorescence images were obtained using Carl Zeiss Axiovert 200M and analyzed by ImageJ program.

2.9. Western blot analysis.

Primary cultured rat VSMCs and HepG2 cells grown in T-25 flasks (80-90% confluent) were harvested in RIPA buffer containing 1X protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc.) on ice. Cell debris was removed by a 15-min centrifugation at 9,800 g. The supernatant was suspended in Laemmli sample buffer and 20 µg (myosin light chain phosphorylation) and 50 µg (caspase-3 activation) protein was electrophoresed in a polyacrylamide mini-slab gel and then transferred onto PVDF membranes in Tris/glycine buffer (25 mM Tris, 192 mM glycine) containing 20% methanol. The extents of each protein were measured with immunoblotting using antibodies against caspase-3 (#9662), myosin light chain (MLC, #3672) and phospho-MLC (#3674) (Cell Signaling Technology Inc.) incubated overnight at 4°C. Immunoreactive bands were visualized by horseradish-peroxidase-conjugated secondary antibody with a Femto chemiluminescence kit (Thermo Fisher Scientific Inc., Rockford, IL) and analyzed using Chemidoc™ XRS system (Bio-Rad laboratories Inc.).

2.10. Cell viability analysis.

Cell viability was determined by the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-dinitrophenyl)-2H-tetrazolium, monosodium salt] assay, using a CCK-8 kit (Dojindo, Japan) according to the manufacturer's instructions. The assay is based on the cleavage of the WST-8 tetrazolium salt to formazan by cellular mitochondrial dehydrogenase. Cell viability was determined following culture in 48-well plates at a seeding density of 4×10^4 cells/well. Cells were treated with 0, 1, 10, or 25 µM of ginsenoside Rg3 for 24 h at 37 °C. WST-8/1-methoxy-phenazine methosulfate solution was added to each well and incubated for 3 h at 37 °C, protected from light exposure. The absorbance at 450 nm was measured. Values are

expressed as a percentage of control cells without ginsenoside Rg3 supplementation.

2.11. Statistical analysis.

All the data were represented by mean $\pm$ SEM of at least three independent experiments. Data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple ranged test or paired student's t-test, using SPSS software (Chicago, IL). In all cases, p value less than 0.05 was considered significant.

3. Results

3.1. Rg3 activates caspase-3 and apoptosis in vascular smooth muscle cells

To examine the mechanism underlying Rg3-induced apoptotic cell death in vascular smooth muscle, caspase-3 activation, a hallmark of apoptosis, was evaluated in rat primary isolated vascular smooth muscle cells (VSMCs) after exposure to Rg3. As shown in Fig. 1A, caspase-3 was activated by Rg3 exposure in a concentration-dependent manner. Consistently, apoptosis was induced by Rg3 at the concentrations equivalent to caspase-3 activation as clarified by TUNEL staining (Fig. 1B). Contractile dysfunction observed as irreversibly decreased tension generation in the aortic ring (Lee, et al., 2010a) was well reproduced in VSMCs as the attenuation of 5-HT-induced MLC phosphorylation, a marker of vascular contraction, by Rg3 (Figs. 1C) (Carrillo-Sepulveda *et al.*, 2010; Lee *et al.*, 2006). Apoptotic cell deaths and contractile dysfunction induced by Rg3 were also exposure-duration dependent (Figs. 1D and 1E) and the patterns were inversely well-correlated with dysfunction preceded the structural damage (Fig. 1F).

3.2. Effects of Rg3 on the integrity of the actin cytoskeleton and mitochondrial function

In order to examine the involvement of actin-cytoskeleton in Rg3-induced contractile dysfunction and apoptotic cell death, we measured the activation of RhoA, which is critical in the maintenance of actin-integrity. As a result, Rg3 significantly attenuated 5-HT-induced RhoA activation in a concentration-dependent manner (Fig. 2A). Immunocytochemistry of F- (red) and G- (green) actin revealed that Rg3 led to the depolymerization of actin and further decreased overall actin levels (Fig. 2B). Instability of actin eventually causes the dissociation of, and subsequent mitochondrial translocation of pro-apoptotic Bmf (Martin *et al.*, 2004).

Indeed, treatment of Rg3 at 25 μ M resulted in mitochondrial translocation of Bmf (Fig. 2C) and subsequent dissipation of mitochondrial membrane potential (Fig. 2D) as shown by immunoblot and JC-1 fluorescence assays, respectively.

3.3. Reversal of Rg3 effects with actin filament stabilization in VSMCs

To illuminate the role of actin destabilization in Rg3-induced apoptotic cell death in VSMCs, an actin-stabilizer, jasplakinolide (Jasp) were co-treated with Rg3. As a result, Jasp effectively prevented Rg3-induced mitochondrial translocation of Bmf (Fig. 3A) and disruption of mitochondrial membrane potential (Fig. 3B). Protection of VSMCs against Rg3-induced apoptotic cell death by Jasp was further confirmed by TUNEL staining (Fig. 3C), reflecting that Rg3 induces apoptotic cell death through actin-cytoskeletal disruption-initiated mitochondrial apoptotic pathway.

3.4. The disruption of actin cytoskeletal integrity may also contribute to the anti-cancer effects of Rg3.

To examine whether the disruption of actin cytoskeleton may contribute to anti-cancer effects of Rg3, we employed hepatocarcinoma cell-line, HepG2 and compared the cytotoxicity profiles with VSMCs. Rg3 treatment resulted in decreased cell viability, which corresponded well in both VSMCs and HepG2 with respect to concentration range (Fig. 4A). Activation of caspase-3 and actin degradation were more prominent in HepG2 (Fig. 4B and 4C) and mitochondrial translocation of Bmf and apoptotic cell death was clearly shown in HepG2 that were significantly reversed by co-treatment of Jasp (Figs. 4D and 4E), reflecting that anti-cancer activities and vascular toxicity of Rg3 can be manifested via a common pathway involving the disruption of actin-integrity.

4. Discussion

Here we demonstrated that ginsenoside Rg3 disrupts the integrity of actin-cytoskeleton leading to contractile dysfunction and apoptotic cell deaths in vascular smooth muscle (Scheme 1). Disruption of the actin cytoskeleton integrity may be mediated at least in part by insufficient RhoA activation which can suppress the actin polymerization and induce F-actin degradation. Dissociation of Bmf from the actin and its mitochondrial translocation primed the dissipation of mitochondrial membrane potential, which triggered the mitochondrial pro-apoptotic pathway. The actin degradation and apoptosis were blocked by an actin stabilizer, jasplakinolide, confirming the central role of the actin disruption in the manifestation of Rg3-induced vascular toxicity.

Of a particular note, the mechanism underlying Rg3-induced vascular toxicity could explain the anti-cancer effects of Rg3, of which results were manifested as pro-apoptotic effects on the cancer cells. Rg3 treatment induces the morphological alteration, mitochondrial damages, and apoptotic cell deaths of cancer cells (Jiang *et al.*, 2011). Previously, anti-cancer mechanisms of Rg3 have been explained by AMPK activation (Yuan, et al., 2010), ROS generation, anti-angiogenesis (Kim *et al.*, 2012), and the suppression of anti-apoptotic NF- κ B (Kim, et al., 2014) and PI3K-Akt signaling (Qiu *et al.*, 2014; Wang *et al.*, 2009). However, these mechanisms can only indirectly allude the possible cytotoxic effects of Rg3 on cancer cells, but could not explain the various phenomena of toxicity of Rg3 on normal cells as well as contractile dysfunction observed in our study. In contrast, actin disruption as demonstrated here can explain well the morphological alteration and contractile dysfunction which may be caused by damaged cytoskeletal integrity, as well as the mitochondrial damages and apoptotic cell deaths which might be from dissociation of pro-apoptotic Bmf from cell membrane and

its mitochondrial translocation, strongly supporting that anti-cancer effects of Rg3 may be explained by the mechanism of actin-targeting anti-cancer agents (ATACs).

Recently ATACs have been drawing attention as a novel class of anti-cancer drugs (Bonello, et al., 2009). Actin degradation and cytoskeletal disruption have previously been suggested as the outcome of caspase activation and degradation of cytoskeleton occurring during apoptotic cell death (Mashima *et al.*, 1999). However, Desouza *et al.* demonstrated that the damage of cytoskeleton, *per se* can trigger apoptosis (Desouza, et al., 2012). Actin and its dynamics are important for the contraction, adhesion and retraction of cancer cells, which are critical in the invasion and the metastasis (Fife *et al.*, 2014; Yamazaki, et al., 2005). Not limited to perturbation of cellular dynamics, Puthalakath *et al.*(2001) demonstrated that actin disruption may induce the mitochondrial apoptotic pathway through dissociating pro-apoptotic Bmf, which is bound with actin via myosin V motor in the intact states. Dissociation of Bmf from actin and its subsequent mitochondrial translocation suppresses the anti-apoptotic Bcl-2, ultimately leading to apoptosis as evidenced by an anti-cancer agent, paclitaxel which is known to induce apoptosis of cancer cells through actin-targeting mechanism (Kutuk *et al.*, 2010). Actin-disruption effects may also be beneficial for the treatment of cancer through impairing angiogenesis through suppressing endothelial proliferation (Thoenes *et al.*, 2008).

ATACs, however, have a selectivity issue, namely, they cannot differentiate the actin of normal cells from that of cancer cells, resulting in severe toxicities as demonstrated during the preclinical development stages (Stehn, et al., 2013). Previously, we demonstrated that Rg3 induces irreversible contractile dysfunction and apoptotic cell deaths of the vascular smooth muscle cells (Lee, et al., 2010a). Along with the well-established anti-cancer effects

of Rg3, we hypothesized that action and toxic mechanisms of Rg3 may involve actin disruption, which can explain both phenomena. Indeed, actin plays pivotal roles in contraction and cellular survival (Gourlay, et al., 2005) and nonspecific perturbation of actin integrity as exemplified by cytochalasin D may result in contractile dysfunction and cell death as Rg3 in our study.

Actin is not static but exists in a dynamic state balanced between polymeric F-actin and monomeric G-actin, which is regulated by actin binding proteins (ABPs) (Lee *et al.*, 2010b) and RhoA (Takai *et al.*, 2001). Kang *et al.* (2014) demonstrated that simvastatin suppresses the action of RhoA, ultimately resulting in vascular toxicity through perturbation of actin integrity. The characteristic of muscular toxicity of simvastatin is strikingly similar with that of Rg3, which also inhibits RhoA activation. In cancer cells, RhoA, is important for cell proliferation and metastasis, which is most probably via actin modulation (Parri *et al.*, 2010; Vega *et al.*, 2008). RhoA is constantly activated in cancer cells by the up-regulation of Rho GDP dissociation inhibitor (GDI) (Wang *et al.*, 2014). Interestingly, Lee *et al.* (2009) demonstrated through proteomics approach that Rg3 down-regulates Rho GDI in cancer cells, which may lead to the attenuation of RhoA activity, supporting that Rg3 may disrupt actin integrity through RhoA-dependent pathway.

Rg3 has been investigated in many anti-cancer clinical trials which may be attributable to the potent anti-cancer effects of Rg3 *in vitro*. Anti-cancer effects of Rg3 *in vitro* are mostly shown at 10 to 300 μ M concentrations which are well-reviewed in a recent paper by Sun *et al.* (Sun, et al., 2017). These concentrations match well with the range we employed (1-25 μ M), reflecting that our results may well occur at the therapeutic concentrations. In clinical trials, Rg3 was generally treated at 20 mg twice daily via oral route

in combination with other anti-cancer therapy for more than 1 month (Li, et al., 2016; Lu, et al., 2008; Zhou, et al., 2016), which corresponds to ~ 1 mg/kg in human. Rg3 is also available as Shenyi capsule or dietary supplements. One of dietary supplements contains 100 mg Rg3 which is intended to be used twice daily for anti-cancer or to improve immunity (SupplementHealthstore, 2017). This dose level falls to 200 mg Rg3 which would be translated to 2-5 mg/kg in human. In a same context with our previous paper (Lee *et al.*, 2010), this level would yield 2-5 $\mu\text{g/mL}$ Rg3 ($2.5 \sim 6 \mu\text{M}$ in total) at blood plasma level, which is not far-fetched from the concentrations (10 ~ 25 μM) we employed. We believe that these facts strongly support the clinical relevance of Rg3 and the concentrations we adopted in enlightening the toxicity of Rg3.

In conclusion, we demonstrated that actin-disruption may be central to Rg3-induced vascular toxicity as manifested by contractile dysfunction and apoptotic cell death. Of a particular note, this toxic mechanism is also shared by the cytotoxicity of Rg3 against cancer cells, which may provide with an important mechanistic insight into understanding anti-cancer effects of Rg3. Interestingly, paclitaxel and cytochalasin D, known to possess ATAC activities are natural products as is Rg3, suggesting that nature-derived anti-cancer agents may have concern for nonspecific toxicity against normal tissues of which point shall be revisited in the future.

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Legends

Figure 1. Effects of Rg3 on contractile dysfunction and apoptosis in VSMCs. (A) p17-active caspase-3 and p-35-procaspase-3 were detected using Western blot analysis. After 24 hr incubation with vehicle (DMSO) or Rg3 (1, 10, 25 μ M) in primary cultured rat VSMCs, concentration dependent effects on caspase-3 activation were determined. (B) After primary VSMCs were incubated with Rg3 (1, 10, 25 μ M) for 24 hr, apoptosis was determined by TUNEL staining. Merged images of TUNEL (green) and Hoechst 33342 (blue) were presented. Intensity of green fluorescence was measured as a parameter of extent of apoptosis. (C) After Rg3 (1, 10, 25 μ M) was treated for 24 hr to primary VSMCs, followed by 5-HT 10^{-5} M stimuli for 10 min, phosphorylation of myosin light chain (MLC) was determined by Western blot analysis. (D) Effects on apoptosis were determined by TUNEL staining, after primary VSMCs were incubated with Rg3 25 μ M for 1, 4, 24 hr. (E) After Rg3 25 μ M was treated for 1, 4, 24 hr to primary VSMCs, followed by 5-HT 10^{-5} M stimuli for 10 min, phosphorylation of myosin light chain (MLC) was determined by Western blot analysis. (F) Time-course effects of Rg3 25 μ M on the inhibition of MLC phosphorylation and apoptosis in primary VSMCs. Data are summarized from (D) and (E). Scale bar = 100 μ m. Values are mean $\pm$ SEM of at least three independent experiments. * represents significant differences from the controls ($p < 0.05$). # represents significant differences from the 5-HT-treated group ($p < 0.05$).

Figure 2. Effects of Rg3 on Contractile Machinery and mitochondria in VSMCs. (A) Concentration dependent effects of Rg3 on RhoA activation were evaluated after treatment of

Rg3 (1, 10, 25 μ M for 1 hr), followed by 5-HT 10^{-5} M stimuli for 10 min to primary cultured rat VSMCs. (B) After Rg3 (1, 10, 25 μ M) was treated for 4 hr, immunocytochemistry of actin was investigated with rhodamine-phalloidin, DNaseI-Alexa 488 and merged images of F-actin(red) and G-actin(green) were obtained. (C) After Rg3 25 μ M was treated for 4 hr, cytosolic and mitochondrial fractions were isolated from VSMCs and the amount of Bmf in each fraction was determined by Western blot. α -Tubulin and cytochrome c oxidase (COX) were used as loading controls for cytosolic and mitochondrial fractions, respectively and Bmf levels for each fraction was normalized with corresponding loading controls. (D) After Rg3 (1, 10, 25 μ M) was treated for 24 hr, mitochondrial outer membrane potential was determined with JC-1 dye. Mitochondrial membrane potential was evaluated as a ratio of red to green fluorescence intensity. Scale bar = 100 μ m. Values are mean $\pm$ SEM of at least three independent experiments. * represents significant differences from the controls ($p < 0.05$).

Figure 3. Reversal of Rg3 effects with actin filament stabilization in VSMCs. (A) After Rg3 25 μ M and jasplakinolide (Jasp) 50 nM were co-incubated for 4 hr, cytosolic and mitochondrial fractions were isolated from VSMCs and the amount of Bmf in each fraction was determined by western blot. (B) After Rg3 25 μ M and jasplakinolide (Jasp) 50 nM were co-incubated for 24 hr, mitochondrial outer membrane potential was determined with JC-1 dye. (C) Apoptosis was determined by TUNEL staining. Merged images of TUNEL (green) and Hoechst 33342 (blue) were presented. Inhibitory effects of Jasp were determined after Rg3 25 μ M with Jasp 50 nM was treated for 24 hr. Scale bar = 100 μ m. Values are mean $\pm$ SEM of at least three independent experiments. * represents significant differences from the controls ($p < 0.05$). # represents significant differences from the Rg3-treated group ($p < 0.05$)

Figure 4. Anti-cancer effects of Rg3 in HepG2 cells. (A) Primary VSMCs and HepG2 Cells were treated with vehicle (DMSO) or Rg3 (1, 10, 25 μ M) and incubated for 24 hr. Cell viability was determined using the WST-8 assay. (B) p17-active caspase-3 and p-35-procaspase-3 were detected using Western blot analysis. After 24 hr incubation with vehicle (DMSO) or Rg3 (1, 10, 25 μ M) in HepG2 cells, concentration dependent effects on caspase-3 activation were determined. (C) After Rg3 (1, 10, 25 μ M) was treated for 4 hr, immunocytochemistry of actin was investigated with rhodamine-phalloidin, DNaseI-Alexa 488 and images of F-actin (red) and G-actin (green) were obtained. The extent of F-actin and G-actin was evaluated with intensity of red and green fluorescence. (D) After Rg3 25 μ M and jasplakinolide (Jasp) 50 nM were co-incubated for 4 hr, cytosolic and mitochondrial fractions were isolated from HepG2 cells and the amount of Bmf in each fraction was determined by western blot. α -Tubulin and cytochrome c oxidase (COX) were used as loading controls for cytosolic and mitochondrial fractions, respectively and Bmf levels for each fraction was normalized with corresponding loading controls. (E) Apoptosis was determined by TUNEL staining. Merged images of TUNEL (green) and Hoechst 33342 (blue) were presented. Inhibitory effects of Jasp were determined after Rg3 25 μ M with Jasp 50 nM was treated for 24 hr to HepG2 cells. Scale bar = 100 μ m. Values are mean $\pm$ SEM of at least three independent experiments. * represents significant differences from the controls. ($p < 0.05$). # represents significant differences from the Rg3-treated group ($p < 0.05$).

Scheme 1. Suggested mechanism of Rg3-induced apoptosis in both normal vascular smooth muscle cells and cancer cells. Ginsenoside Rg3 (Rg3) disrupts actin integrity, which

results in contractile dysfunction. Disruption of actin integrity initiates translocation of Bmf followed by mitochondrial apoptotic pathway, ultimately leading to vascular toxicity. Disruption of actin integrity is the key role of Rg3-induced apoptosis which can explain both anti-cancer effects and vascular toxicity.

Abstract

Ginseng and its major active ingredients, ginsenosides are widely consumed for various health problems including cancer without a special precaution. Previously, we demonstrated that Rg3, one of ginsenosides with a potent anti-cancer activity, may cause contractile dysfunction, and structural damage on vascular smooth muscle through apoptotic cell death. However, the mechanism underlying Rg3-induced vascular toxicity was not fully elucidated. Here we demonstrated that the vascular toxicity of Rg3 occurs through actin disruption and Bmf-initiated mitochondrial apoptotic pathway, the mechanism which may contribute to its anti-cancer effects. In rat primary vascular smooth muscle cells, Rg3 induced apoptosis and contractile dysfunction as determined by caspase-3 activation, TUNEL staining, and insufficient myosin light chain phosphorylation. Rg3 induced actin degradation with Rho A inactivation, which was followed by mitochondrial translocation of Bmf and dissociation of mitochondrial membrane potential (ϕ). Pre-treatment of jasplakinolide, an inhibitor of actin depolymerization, significantly blocked Rg3-induced ϕ dissipation and apoptosis. Notably, Rg3 induced actin disruption in hepatoma cell line, HepG2, which could be reversed by jasplakinolide, reflecting that the same mechanism may contribute to the anti-cancer effects of Rg3 at least in part. Collectively, these results demonstrated that Rg3 may induce vascular toxicity as well as anti-cancer effects through disrupting actin, which may alert the potential toxicity associated with inadvertent use of ginseng and its products.

Key words: ginseng; ginsenoside Rg3; actin cytoskeleton; vascular toxicity; apoptosis; actin-targeting anti-cancer agents

Ginsenoside Rg3 disrupts actin-cytoskeletal integrity leading to contractile dysfunction and apoptotic cell death in vascular smooth muscle

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Competing Financial Interests Declaration: None

Abstract

Ginseng and its major active ingredients, ginsenosides are widely consumed for various health benefits including the prevention or treatment of cancer without any special precaution. Previously, we demonstrated that Rg3, one of ginsenosides with a potent anti-cancer activity, may cause contractile dysfunction, and structural damage against normal vascular smooth muscle through apoptotic cell death. However, mechanism underlying the vascular toxicity of Rg3 was not fully elucidated. Here we demonstrated that the vascular toxicity of Rg3 occurs through actin disruption and Bmf-initiated mitochondrial apoptotic pathway, the mechanism which may be shared with its anti-cancer effects. In normal rat primary vascular smooth muscle cells, Rg3 induced apoptosis and contractile dysfunction as determined by caspase-3 activation, TUNEL staining, and insufficient myosin light chain phosphorylation. Rg3 induced actin degradation with RhoA inactivation, followed by mitochondrial translocation of Bmf and dissociation of mitochondrial membrane potential (ϕ). Pre-treatment of jasplakinolide, an inhibitor of actin depolymerization, significantly blocked Rg3-induced ϕ dissipation and apoptosis. Notably, Rg3 also induced actin disruption in hepatoma cell line, HepG2, which was reversed by jasplakinolide, confirming that the anti-cancer effects of Rg3 shares the mechanism at least in part. Collectively, these results demonstrated that Rg3 may induce vascular toxicity as well as anti-cancer effects through disrupting actin, suggesting the potential toxicity associated with inadvertent use of ginseng and its products.

Key words: ginseng; ginsenoside Rg3; actin cytoskeleton; vascular toxicity; apoptosis; actin-targeting anti-cancer agents

1. Introduction

Panax ginseng C. A. meyer, ginseng is a symbolic herbal medicine heavily consumed in Eastern Asian countries that include Korea, China and even US as dietary supplement, tonics, alternative medicines and even as panacea (Baeg *et al.*, 2013). Diverse biological activities have been reported for ginseng extract and its ingredients, which include anti-aging, anti-cancer, anti-hypertensive, anti-inflammatory, and anti-oxidant activities (Karmazyn *et al.*, 2011). These biological activities of ginseng are mostly attributable to ginsenosides, a major saponic component enriched in it. Along with the potent therapeutic effects, a concern over the potential toxicity of ginseng and ginsenosides has been raised. It is reported that long-term consumption of ginseng at high doses is not without toxicities (Paik *et al.*, 2015). Siegel conducted two year studies in the subjects consuming ginseng extracts, which revealed frequent side effects like morning diarrhea, skin eruption, nervousness, sleeplessness, hypertension, edema, decreased appetite, depression, and hypotension (Siegel, 1979). Especially, ginseng products are widely consumed for the improvement of cardiovascular function (Stavro *et al.*, 2006) but the potential **vascular toxicity** of ginseng and ginsenosides is drawing attention with respect to hypertension, hypotension and vascular dysfunction (Ernst, 2002; Lee *et al.*, 2010a; Siegel, 1980).

Despite many lines of experimental and clinical evidence for the **vascular toxicity** of ginseng and ginsenosides, detailed mechanistic explanation has been rarely provided. Recently, we demonstrated that Rg3, a ginsenoside with diverse potent biological activities including the anti-hypertensive (Kim *et al.*, 1999; Kim *et al.*, 2003), neuroprotective (Tian *et al.*, 2005), anti-cancer (Sun *et al.*, 2017), anti-metastatic (Choi *et al.*, 2013) and anti-proliferative activities (He *et al.*, 2011), can inflict irreversible damages on the contractile

function of the vascular smooth muscle and eventually lead to the apoptosis of smooth muscle cells and vascular remodeling *in vivo* (Lee, et al., 2010a). Especially, Rg3 is known to have anti-metastatic and anti-proliferative effects (Christensen, 2009; Helms, 2004). It is widely consumed as dietary supplements for various health benefits including the prevention of cancers (GuangzhouEndlessBiotech, 2017; SupplementHealthstore, 2017) and has even examined in clinical trials for anti-cancer effects as well (Li *et al.*, 2016; Lu *et al.*, 2008; Zhou *et al.*, 2016). We hypothesized that the vascular toxicity of Rg3 could be linked to its anti-cancer activities since anti-proliferative and anti-metastatic effects may appear as toxicity against normal tissues. However, conventionally known molecular mechanisms for the anti-cancer activities of Rg3, like ROS generation, NF- κ B, ERK activation (Choi, *et al.*, 2013; Kim *et al.*, 2014), and AMPK-mediated mitochondrial apoptotic pathways (Yuan *et al.*, 2010) could not fully explain the vascular toxicity of Rg3 with respect to contractile dysfunction.

Recently, a new class of anti-cancer agents with a novel anti-proliferative mechanism has been introduced, which targets the actin-cytoskeleton of cancer cells (Bonello *et al.*, 2009). These actin-targeting anti-cancer agents (ATACs) attack the actin cytoskeleton, which triggers the disintegration of actin, and the subsequent dissociation and mitochondrial translocation of actin-associated Bmf, a pro-apoptotic BH-3 only Bcl family protein. Pro-apoptotic Bmf, then initiates the apoptotic process in the mitochondria of the affected cancer cells (Desouza *et al.*, 2012; Puthalakath *et al.*, 2001). ATACs have safety issues however, since the actin cytoskeleton of cancer cells could not be differentiated from that of normal cells (Stehn *et al.*, 2013), which plays a pivotal role in the function or survival of cells (Franklin-Tong *et al.*, 2008; Gourlay *et al.*, 2005; Yamazaki *et al.*, 2005).

Especially for the muscle tissues where actin is critical for the generation of contractile forces (Reho *et al.*, 2014), ATACs may be highly toxic, of which point is well corroborated by the reports of their adverse effects against heart, vascular smooth muscle and diaphragm (Stehn *et al.*, 2006). Cytochalasin, a representative ATAC which impairs actin polymerization and thereby, disintegrates actin structure, suppresses the proliferation and invasion of cancer cells (Trendowski, 2015), is well known to induce cardiac and vascular toxicity through causing contractile dysfunction via inhibiting MLC phosphorylation (Bruijns *et al.*, 2001).

The anti-cancer mechanism of ATACs gave a clue to us for the explanation of the vascular toxicity of Rg3 since the impairment of actin cytoskeleton can induce both contractile dysfunction and apoptotic cell death. Here, we examined whether Rg3 can induce toxicity against vascular smooth muscle in the aspect of actin-disruption and subsequent activation of Bmf-initiated apoptosis. In addition, we also investigated whether Rg3 could affect the actin cytoskeleton of cancer cell-line to explore if the anti-cancer mechanism of Rg3 converges with that of vascular toxicity.

2. Materials & Methods

2.1. Reagents.

The following chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO): ginsenoside Rg3, serotonin hydrochloride (5-HT) and dimethyl sulfoxide (DMSO). Jaspilakinolide was purchased from Molecular Probes Inc. (Eugene, OR) and reagents and media used in cell culture were purchased from Gibco Co. (Carlsbad, CA). All other reagents used were of the highest purity available.

2.2. Animals.

Male Sprague-Dawley (SD) rats (SamTako, Korea) weighing 150-170 g were used in experiment for primary culture of vascular smooth muscle cells (VSMCs). Before the experiments, the animals were acclimated for 1 week under controlled laboratory conditions of temperature at $22 \pm 2^{\circ}\text{C}$ and humidity at $55 \pm 5\%$ with 12 hr light/12 hr dark cycle. Normal diet (Cargill Agri Purina, Inc., Korea) and water were provided *ad libitum*. All protocols were approved by the Ethics Committee of the Animal Service Center at Seoul National University.

2.3. Cell culture.

After thoracic aorta was isolated from SD rats, the endothelium was removed by gently rubbing the intimal surface, and adventitia was removed using collagenase (150 U/mL, Worthington Biochemical Co., Lakewood, NJ). The thoracic aorta was chopped finely and vascular smooth muscle cells (VSMCs) were liberated from the tissue using collagenase (150 U/mL) and elastase (1.5 U/mL, Worthington Biochemical Co.). VSMCs were incubated in

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in a 95% air/5% CO₂ and 37°C incubator. VSMCs at passage 1-3 were used for experiments. The human HepG2 cell line was obtained from American Type Culture Collection (Manassas, VA). HepG2 cells were incubated in low glucose DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in a 95% air/5% CO₂ and 37°C incubator. Medium was exchanged every 2-3 days.

2.4. Terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining of VSMCs and HepG2 cells.

For TUNEL staining in VSMCs and HepG2 cells, cells (2x10⁵ cells) were grown on collagen coated coverslips in 6-well plates, incubated with ginsenoside Rg3 with or without jasplakinolide 50 nM and then TUNEL stained using Click-iT[®] TUNEL Alexa Fluor[®] Imaging Kit (Molecular Probes Inc., Eugene, OR), according to the manufacturer's instruction. In brief, cells were fixed in 4% formaldehyde in phosphate buffered saline (PBS; 1 mM KH₂PO₄, 154 mM NaCl, 3 mM Na₂HPO₄, pH 7.4) for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature and then incubated with TUNEL reaction mixture containing TdT in a humidified chamber for 60 min at 37°C. After TUNEL reaction, Click-iT reaction mixture containing Alexa 488 azide for 30 min at room temperature, protected from light exposure. Hoechst 33342 was counterstained to examine nuclear morphology. After staining, fluorescent images were obtained using Axiovert 200M fluorescence microscope (Carl Zeiss, Jena, Germany). Intensity of fluorescence was analyzed by ImageJ program (NIH, Bethesda, MD).

2.5. Determination of RhoA activation.

RhoA activity was measured using a commercially available Rho/Rac-1/Cdc42 activation assay kit (Cytoskeleton Inc., Denver, CO) as instructed by the manufacturer. Primary cultured rat VSMCs grown in T-25 flasks (80-90% confluent) were incubated with ginsenoside Rg3 (0, 1, 10, 25 μ M) for 1 hr and then, stimulated with 5-HT 10^{-5} M for 10 min. The cells were harvested in lysis buffer containing protease inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL) on ice. Cell debris was removed by a 15 min centrifugation at 9,800 g, and the supernatant was incubated with rhotekin-RBD beads specifically recognizing active RhoA for 1 hr at 4°C. Beads were washed, and the precipitated active RhoA were detected by western blotting and incubation with specific monoclonal antibody against RhoA (#ARH03, Cytoskeleton Inc., Denver, CO) overnight at 4°C.

2.6. Visualization of actin filament in VSMCs and HepG2 cells.

VSMCs and HepG2 cells (2×10^5 cells) grown on collagen coated coverslip in 6-well plates were incubated with ginsenoside Rg3 (0, 1, 10, 25 μ M) for 4 hr. Cells were fixed with 4% formaldehyde in PBS for 10 min, followed by permeabilization with 0.5% Triton X-100 for 5 min and blocking with 2% bovine serum albumin (BSA) for 1 hr at room temperature. To visualize the actin filament, rhodamine conjugated phalloidin (F-actin) and Alexa 488 conjugated DNase I (G-actin) (Molecular Probes Inc.) was incubated for 1 hr at room temperature, protected from light exposure. Fluorescence images were obtained using Carl Zeiss Axiovert 200M and analyzed by ImageJ program.

2.7. Mitochondrial fractionation and analysis of Bmf translocation.

After cultured rat VSMCs and HepG2 cells grown in 100 mm dish (80-90% confluent) were incubated with ginsenoside Rg3 25 μ M with or without jasplakinolide 50 nM for 4 hr, cytosolic and mitochondrial fractions of VSMCs and HepG2 cells were isolated using a mitochondria isolation kit (Thermo Fisher Scientific Inc., Rockford, IL) according to manufacturer's instruction. Subcellular protein extracts were suspended in Laemmli sample buffer (Bio-Rad, Hercules, CA) and 10 μ g protein of cytosolic fraction and 4 μ g of membrane fraction were subjected to western blot analysis. Blots were detected by incubation with primary antibody against Bmf (#5889, Cell Signaling Technology Inc., Danvers, MA) overnight at 4°C. Antibodies against α -tubulin (#2125) and cytochrome c oxidase (COX) IV (#4844) (Cell Signaling Technology Inc.) were used as loading controls for cytosolic and mitochondrial proteins, respectively.

2.8. Determination of mitochondrial membrane potential.

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were determined with the cationic fluorochrome JC-1 (Sigma-Aldrich Co.), which accumulates in mitochondria as red fluorescent aggregates at intact $\Delta\Psi_m$, whereas exists in green fluorescent monomeric form at decreased $\Delta\Psi_m$. VSMCs (2×10^5 cells) were grown in 6-well plates with collagen coated coverslip and treated with ginsenoside Rg3 with or without jasplakinolide 50 nM for 24 hr. VSMCs were incubated with JC-1 (final 2 μ g/ml) for 15 min at 37°C and fluorescence images were obtained using Carl Zeiss Axiovert 200M and analyzed by ImageJ program.

2.9. Western blot analysis.

Primary cultured rat VSMCs and HepG2 cells grown in T-25 flasks (80-90% confluent) were harvested in RIPA buffer containing 1X protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc.) on ice. Cell debris was removed by a 15-min centrifugation at 9,800 g. The supernatant was suspended in Laemmli sample buffer and 20 µg (myosin light chain phosphorylation) and 50 µg (caspase-3 activation) protein was electrophoresed in a polyacrylamide mini-slab gel and then transferred onto PVDF membranes in Tris/glycine buffer (25 mM Tris, 192 mM glycine) containing 20% methanol. The extents of each protein were measured with immunoblotting using antibodies against caspase-3 (#9662), myosin light chain (MLC, #3672) and phospho-MLC (#3674) (Cell Signaling Technology Inc.) incubated overnight at 4°C. Immunoreactive bands were visualized by horseradish-peroxidase-conjugated secondary antibody with a Femto chemiluminescence kit (Thermo Fisher Scientific Inc., Rockford, IL) and analyzed using Chemidoc™ XRS system (Bio-Rad laboratories Inc.).

2.10. Cell viability analysis.

Cell viability was determined by the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-dinitrophenyl)-2H-tetrazolium, monosodium salt] assay, using a CCK-8 kit (Dojindo, Japan) according to the manufacturer's instructions. The assay is based on the cleavage of the WST-8 tetrazolium salt to formazan by cellular mitochondrial dehydrogenase. Cell viability was determined following culture in 48-well plates at a seeding density of 4×10^4 cells/well. Cells were treated with 0, 1, 10, or 25 µM of ginsenoside Rg3 for 24 h at 37 °C. WST-8/1-methoxy-phenazine methosulfate solution was added to each well and incubated for 3 h at 37 °C, protected from light exposure. The absorbance at 450 nm was measured. Values are

expressed as a percentage of control cells without ginsenoside Rg3 supplementation.

2.11. Statistical analysis.

All the data were represented by mean $\pm$ SEM of at least three independent experiments. Data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple ranged test or paired student's t-test, using SPSS software (Chicago, IL). In all cases, p value less than 0.05 was considered significant.

3. Results

3.1. Rg3 activates caspase-3 and apoptosis in vascular smooth muscle cells

To examine the mechanism underlying Rg3-induced apoptotic cell death in vascular smooth muscle, caspase-3 activation, a hallmark of apoptosis, was evaluated in rat primary isolated vascular smooth muscle cells (VSMCs) after exposure to Rg3. As shown in Fig. 1A, caspase-3 was activated by Rg3 exposure in a concentration-dependent manner. Consistently, apoptosis was induced by Rg3 at the concentrations equivalent to caspase-3 activation as clarified by TUNEL staining (Fig. 1B). Contractile dysfunction observed as irreversibly decreased tension generation in the aortic ring (Lee, et al., 2010a) was well reproduced in VSMCs as the attenuation of 5-HT-induced MLC phosphorylation, a marker of vascular contraction, by Rg3 (Figs. 1C) (Carrillo-Sepulveda *et al.*, 2010; Lee *et al.*, 2006). Apoptotic cell deaths and contractile dysfunction induced by Rg3 were also exposure-duration dependent (Figs. 1D and 1E) and the patterns were inversely well-correlated with dysfunction preceded the structural damage (Fig. 1F).

3.2. Effects of Rg3 on the integrity of the actin cytoskeleton and mitochondrial function

In order to examine the involvement of actin-cytoskeleton in Rg3-induced contractile dysfunction and apoptotic cell death, we measured the activation of RhoA, which is critical in the maintenance of actin-integrity. As a result, Rg3 significantly attenuated 5-HT-induced RhoA activation in a concentration-dependent manner (Fig. 2A). Immunocytochemistry of F- (red) and G- (green) actin revealed that Rg3 led to the depolymerization of actin and further decreased overall actin levels (Fig. 2B). Instability of actin eventually causes the dissociation of, and subsequent mitochondrial translocation of pro-apoptotic Bmf (Martin *et al.*, 2004).

Indeed, treatment of Rg3 at 25 μ M resulted in mitochondrial translocation of Bmf (Fig. 2C) and subsequent dissipation of mitochondrial membrane potential (Fig. 2D) as shown by immunoblot and JC-1 fluorescence assays, respectively.

3.3. Reversal of Rg3 effects with actin filament stabilization in VSMCs

To illuminate the role of actin destabilization in Rg3-induced apoptotic cell death in VSMCs, an actin-stabilizer, jasplakinolide (Jasp) were co-treated with Rg3. As a result, Jasp effectively prevented Rg3-induced mitochondrial translocation of Bmf (Fig. 3A) and disruption of mitochondrial membrane potential (Fig. 3B). Protection of VSMCs against Rg3-induced apoptotic cell death by Jasp was further confirmed by TUNEL staining (Fig. 3C), reflecting that Rg3 induces apoptotic cell death through actin-cytoskeletal disruption-initiated mitochondrial apoptotic pathway.

3.4. The disruption of actin cytoskeletal integrity may also contribute to the anti-cancer effects of Rg3.

To examine whether the disruption of actin cytoskeleton may contribute to anti-cancer effects of Rg3, we employed hepatocarcinoma cell-line, HepG2 and compared the cytotoxicity profiles with VSMCs. Rg3 treatment resulted in decreased cell viability, which corresponded well in both VSMCs and HepG2 with respect to concentration range (Fig. 4A). Activation of caspase-3 and actin degradation were more prominent in HepG2 (Fig. 4B and 4C) and mitochondrial translocation of Bmf and apoptotic cell death was clearly shown in HepG2 that were significantly reversed by co-treatment of Jasp (Figs. 4D and 4E), reflecting that anti-cancer activities and vascular toxicity of Rg3 can be manifested via a common pathway involving the disruption of actin-integrity.

4. Discussion

Here we demonstrated that ginsenoside Rg3 disrupts the integrity of actin-cytoskeleton leading to contractile dysfunction and apoptotic cell deaths in vascular smooth muscle (Scheme 1). Disruption of the actin cytoskeleton integrity may be mediated at least in part by insufficient RhoA activation which can suppress the actin polymerization and induce F-actin degradation. Dissociation of Bmf from the actin and its mitochondrial translocation primed the dissipation of mitochondrial membrane potential, which triggered the mitochondrial pro-apoptotic pathway. The actin degradation and apoptosis were blocked by an actin stabilizer, jasplakinolide, confirming the central role of the actin disruption in the manifestation of Rg3-induced vascular toxicity.

Of a particular note, the mechanism underlying Rg3-induced vascular toxicity could explain the anti-cancer effects of Rg3, of which results were manifested as pro-apoptotic effects on the cancer cells. Rg3 treatment induces the morphological alteration, mitochondrial damages, and apoptotic cell deaths of cancer cells (Jiang *et al.*, 2011). Previously, anti-cancer mechanisms of Rg3 have been explained by AMPK activation (Yuan, et al., 2010), ROS generation, anti-angiogenesis (Kim *et al.*, 2012), and the suppression of anti-apoptotic NF- κ B (Kim, et al., 2014) and PI3K-Akt signaling (Qiu *et al.*, 2014; Wang *et al.*, 2009). However, these mechanisms can only indirectly allude the possible cytotoxic effects of Rg3 on cancer cells, but could not explain the various phenomena of toxicity of Rg3 on normal cells as well as contractile dysfunction observed in our study. In contrast, actin disruption as demonstrated here can explain well the morphological alteration and contractile dysfunction which may be caused by damaged cytoskeletal integrity, as well as the mitochondrial damages and apoptotic cell deaths which might be from dissociation of pro-apoptotic Bmf from cell membrane and

its mitochondrial translocation, strongly supporting that anti-cancer effects of Rg3 may be explained by the mechanism of actin-targeting anti-cancer agents (ATACs).

Recently ATACs have been drawing attention as a novel class of anti-cancer drugs (Bonello, et al., 2009). Actin degradation and cytoskeletal disruption have previously been suggested as the outcome of caspase activation and degradation of cytoskeleton occurring during apoptotic cell death (Mashima *et al.*, 1999). However, Desouza *et al.* demonstrated that the damage of cytoskeleton, *per se* can trigger apoptosis (Desouza, et al., 2012). Actin and its dynamics are important for the contraction, adhesion and retraction of cancer cells, which are critical in the invasion and the metastasis (Fife *et al.*, 2014; Yamazaki, et al., 2005). Not limited to perturbation of cellular dynamics, Puthalakath *et al.*(2001) demonstrated that actin disruption may induce the mitochondrial apoptotic pathway through dissociating pro-apoptotic Bmf, which is bound with actin via myosin V motor in the intact states. Dissociation of Bmf from actin and its subsequent mitochondrial translocation suppresses the anti-apoptotic Bcl-2, ultimately leading to apoptosis as evidenced by an anti-cancer agent, paclitaxel which is known to induce apoptosis of cancer cells through actin-targeting mechanism (Kutuk *et al.*, 2010). Actin-disruption effects may also be beneficial for the treatment of cancer through impairing angiogenesis through suppressing endothelial proliferation (Thoenes *et al.*, 2008).

ATACs, however, have a selectivity issue, namely, they cannot differentiate the actin of normal cells from that of cancer cells, resulting in severe toxicities as demonstrated during the preclinical development stages (Stehn, et al., 2013). Previously, we demonstrated that Rg3 induces irreversible contractile dysfunction and apoptotic cell deaths of the vascular smooth muscle cells (Lee, et al., 2010a). Along with the well-established anti-cancer effects

of Rg3, we hypothesized that action and toxic mechanisms of Rg3 may involve actin disruption, which can explain both phenomena. Indeed, actin plays pivotal roles in contraction and cellular survival (Gourlay, et al., 2005) and nonspecific perturbation of actin integrity as exemplified by cytochalasin D may result in contractile dysfunction and cell death as Rg3 in our study.

Actin is not static but exists in a dynamic state balanced between polymeric F-actin and monomeric G-actin, which is regulated by actin binding proteins (ABPs) (Lee *et al.*, 2010b) and RhoA (Takai *et al.*, 2001). Kang *et al.* (2014) demonstrated that simvastatin suppresses the action of RhoA, ultimately resulting in vascular toxicity through perturbation of actin integrity. The characteristic of muscular toxicity of simvastatin is strikingly similar with that of Rg3, which also inhibits RhoA activation. In cancer cells, RhoA, is important for cell proliferation and metastasis, which is most probably via actin modulation (Parri *et al.*, 2010; Vega *et al.*, 2008). RhoA is constantly activated in cancer cells by the up-regulation of Rho GDP dissociation inhibitor (GDI) (Wang *et al.*, 2014). Interestingly, Lee *et al.* (2009) demonstrated through proteomics approach that Rg3 down-regulates Rho GDI in cancer cells, which may lead to the attenuation of RhoA activity, supporting that Rg3 may disrupt actin integrity through RhoA-dependent pathway.

Rg3 has been investigated in many anti-cancer clinical trials which may be attributable to the potent anti-cancer effects of Rg3 *in vitro*. Anti-cancer effects of Rg3 *in vitro* are mostly shown at 10 to 300 μ M concentrations which are well-reviewed in a recent paper by Sun *et al.* (Sun, et al., 2017). These concentrations match well with the range we employed (1-25 μ M), reflecting that our results may well occur at the therapeutic concentrations. In clinical trials, Rg3 was generally treated at 20 mg twice daily via oral route

in combination with other anti-cancer therapy for more than 1 month (Li, et al., 2016; Lu, et al., 2008; Zhou, et al., 2016), which corresponds to ~ 1 mg/kg in human. Rg3 is also available as Shenyi capsule or dietary supplements. One of dietary supplements contains 100 mg Rg3 which is intended to be used twice daily for anti-cancer or to improve immunity (SupplementHealthstore, 2017). This dose level falls to 200 mg Rg3 which would be translated to 2-5 mg/kg in human. In a same context with our previous paper (Lee *et al.*, 2010), this level would yield 2-5 $\mu\text{g/mL}$ Rg3 ($2.5 \sim 6 \mu\text{M}$ in total) at blood plasma level, which is not far-fetched from the concentrations (10 ~ 25 μM) we employed. We believe that these facts strongly support the clinical relevance of Rg3 and the concentrations we adopted in enlightening the toxicity of Rg3.

In conclusion, we demonstrated that actin-disruption may be central to Rg3-induced vascular toxicity as manifested by contractile dysfunction and apoptotic cell death. Of a particular note, this toxic mechanism is also shared by the cytotoxicity of Rg3 against cancer cells, which may provide with an important mechanistic insight into understanding anti-cancer effects of Rg3. Interestingly, paclitaxel and cytochalasin D, known to possess ATAC activities are natural products as is Rg3, suggesting that nature-derived anti-cancer agents may have concern for nonspecific toxicity against normal tissues of which point shall be revisited in the future.

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Legends

Figure 1. Effects of Rg3 on contractile dysfunction and apoptosis in VSMCs. (A) p17-active caspase-3 and p-35-procaspase-3 were detected using Western blot analysis. After 24 hr incubation with vehicle (DMSO) or Rg3 (1, 10, 25 μ M) in primary cultured rat VSMCs, concentration dependent effects on caspase-3 activation were determined. (B) After primary VSMCs were incubated with Rg3 (1, 10, 25 μ M) for 24 hr, apoptosis was determined by TUNEL staining. Merged images of TUNEL (green) and Hoechst 33342 (blue) were presented. Intensity of green fluorescence was measured as a parameter of extent of apoptosis. (C) After Rg3 (1, 10, 25 μ M) was treated for 24 hr to primary VSMCs, followed by 5-HT 10^{-5} M stimuli for 10 min, phosphorylation of myosin light chain (MLC) was determined by Western blot analysis. (D) Effects on apoptosis were determined by TUNEL staining, after primary VSMCs were incubated with Rg3 25 μ M for 1, 4, 24 hr. (E) After Rg3 25 μ M was treated for 1, 4, 24 hr to primary VSMCs, followed by 5-HT 10^{-5} M stimuli for 10 min, phosphorylation of myosin light chain (MLC) was determined by Western blot analysis. (F) Time-course effects of Rg3 25 μ M on the inhibition of MLC phosphorylation and apoptosis in primary VSMCs. Data are summarized from (D) and (E). Scale bar = 100 μ m. Values are mean $\pm$ SEM of at least three independent experiments. * represents significant differences from the controls ($p < 0.05$). # represents significant differences from the 5-HT-treated group ($p < 0.05$).

Figure 2. Effects of Rg3 on Contractile Machinery and mitochondria in VSMCs. (A) Concentration dependent effects of Rg3 on RhoA activation were evaluated after treatment of

Rg3 (1, 10, 25 μ M for 1 hr), followed by 5-HT 10^{-5} M stimuli for 10 min to primary cultured rat VSMCs. (B) After Rg3 (1, 10, 25 μ M) was treated for 4 hr, immunocytochemistry of actin was investigated with rhodamine-phalloidin, DNaseI-Alexa 488 and merged images of F-actin(red) and G-actin(green) were obtained. (C) After Rg3 25 μ M was treated for 4 hr, cytosolic and mitochondrial fractions were isolated from VSMCs and the amount of Bmf in each fraction was determined by Western blot. α -Tubulin and cytochrome c oxidase (COX) were used as loading controls for cytosolic and mitochondrial fractions, respectively and Bmf levels for each fraction was normalized with corresponding loading controls. (D) After Rg3 (1, 10, 25 μ M) was treated for 24 hr, mitochondrial outer membrane potential was determined with JC-1 dye. Mitochondrial membrane potential was evaluated as a ratio of red to green fluorescence intensity. Scale bar = 100 μ m. Values are mean $\pm$ SEM of at least three independent experiments. * represents significant differences from the controls ($p < 0.05$).

Figure 3. Reversal of Rg3 effects with actin filament stabilization in VSMCs. (A) After Rg3 25 μ M and jasplakinolide (Jasp) 50 nM were co-incubated for 4 hr, cytosolic and mitochondrial fractions were isolated from VSMCs and the amount of Bmf in each fraction was determined by western blot. (B) After Rg3 25 μ M and jasplakinolide (Jasp) 50 nM were co-incubated for 24 hr, mitochondrial outer membrane potential was determined with JC-1 dye. (C) Apoptosis was determined by TUNEL staining. Merged images of TUNEL (green) and Hoechst 33342 (blue) were presented. Inhibitory effects of Jasp were determined after Rg3 25 μ M with Jasp 50 nM was treated for 24 hr. Scale bar = 100 μ m. Values are mean $\pm$ SEM of at least three independent experiments. * represents significant differences from the controls ($p < 0.05$). # represents significant differences from the Rg3-treated group ($p < 0.05$)

Figure 4. Anti-cancer effects of Rg3 in HepG2 cells. (A) Primary VSMCs and HepG2 Cells were treated with vehicle (DMSO) or Rg3 (1, 10, 25 μ M) and incubated for 24 hr. Cell viability was determined using the WST-8 assay. (B) p17-active caspase-3 and p-35-procaspase-3 were detected using Western blot analysis. After 24 hr incubation with vehicle (DMSO) or Rg3 (1, 10, 25 μ M) in HepG2 cells, concentration dependent effects on caspase-3 activation were determined. (C) After Rg3 (1, 10, 25 μ M) was treated for 4 hr, immunocytochemistry of actin was investigated with rhodamine-phalloidin, DNaseI-Alexa 488 and images of F-actin (red) and G-actin (green) were obtained. The extent of F-actin and G-actin was evaluated with intensity of red and green fluorescence. (D) After Rg3 25 μ M and jasplakinolide (Jasp) 50 nM were co-incubated for 4 hr, cytosolic and mitochondrial fractions were isolated from HepG2 cells and the amount of Bmf in each fraction was determined by western blot. α -Tubulin and cytochrome c oxidase (COX) were used as loading controls for cytosolic and mitochondrial fractions, respectively and Bmf levels for each fraction was normalized with corresponding loading controls. (E) Apoptosis was determined by TUNEL staining. Merged images of TUNEL (green) and Hoechst 33342 (blue) were presented. Inhibitory effects of Jasp were determined after Rg3 25 μ M with Jasp 50 nM was treated for 24 hr to HepG2 cells. Scale bar = 100 μ m. Values are mean $\pm$ SEM of at least three independent experiments. * represents significant differences from the controls. ($p < 0.05$). # represents significant differences from the Rg3-treated group ($p < 0.05$).

Scheme 1. Suggested mechanism of Rg3-induced apoptosis in both normal vascular smooth muscle cells and cancer cells. Ginsenoside Rg3 (Rg3) disrupts actin integrity, which

results in contractile dysfunction. Disruption of actin integrity initiates translocation of Bmf followed by mitochondrial apoptotic pathway, ultimately leading to vascular toxicity. Disruption of actin integrity is the key role of Rg3-induced apoptosis which can explain both anti-cancer effects and vascular toxicity.

Figure 1
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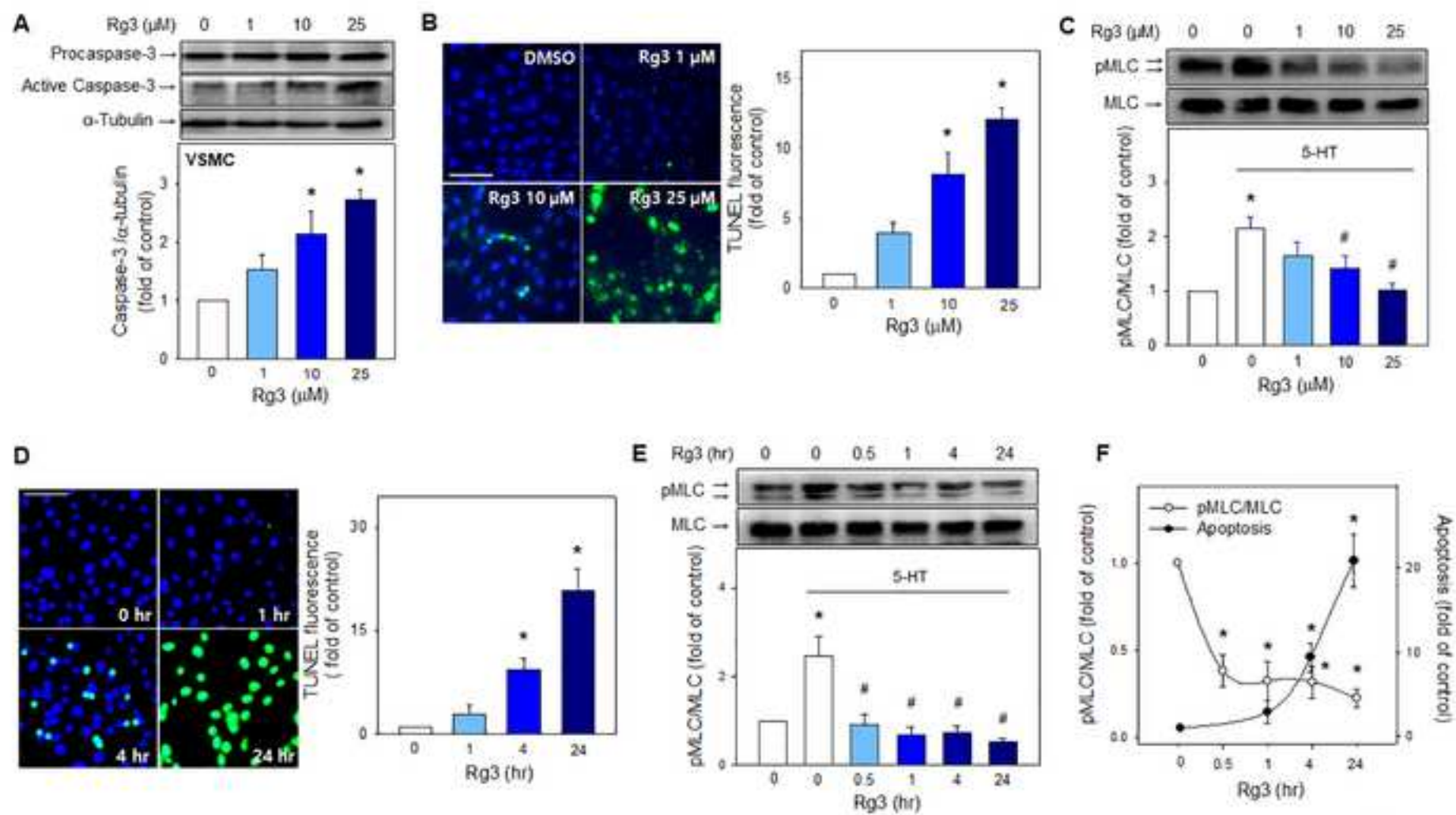


Figure 1

Figure 2
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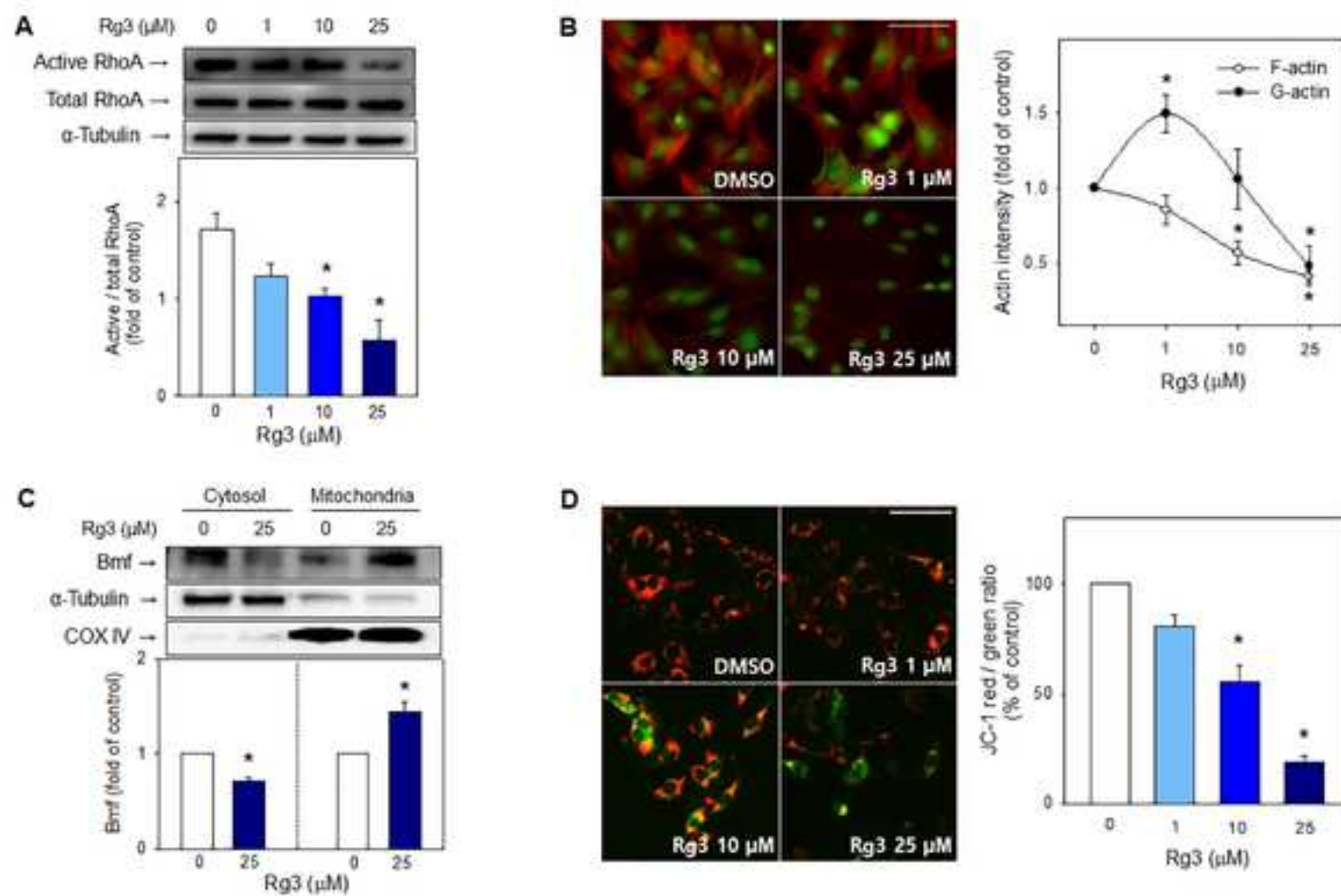


Figure 2

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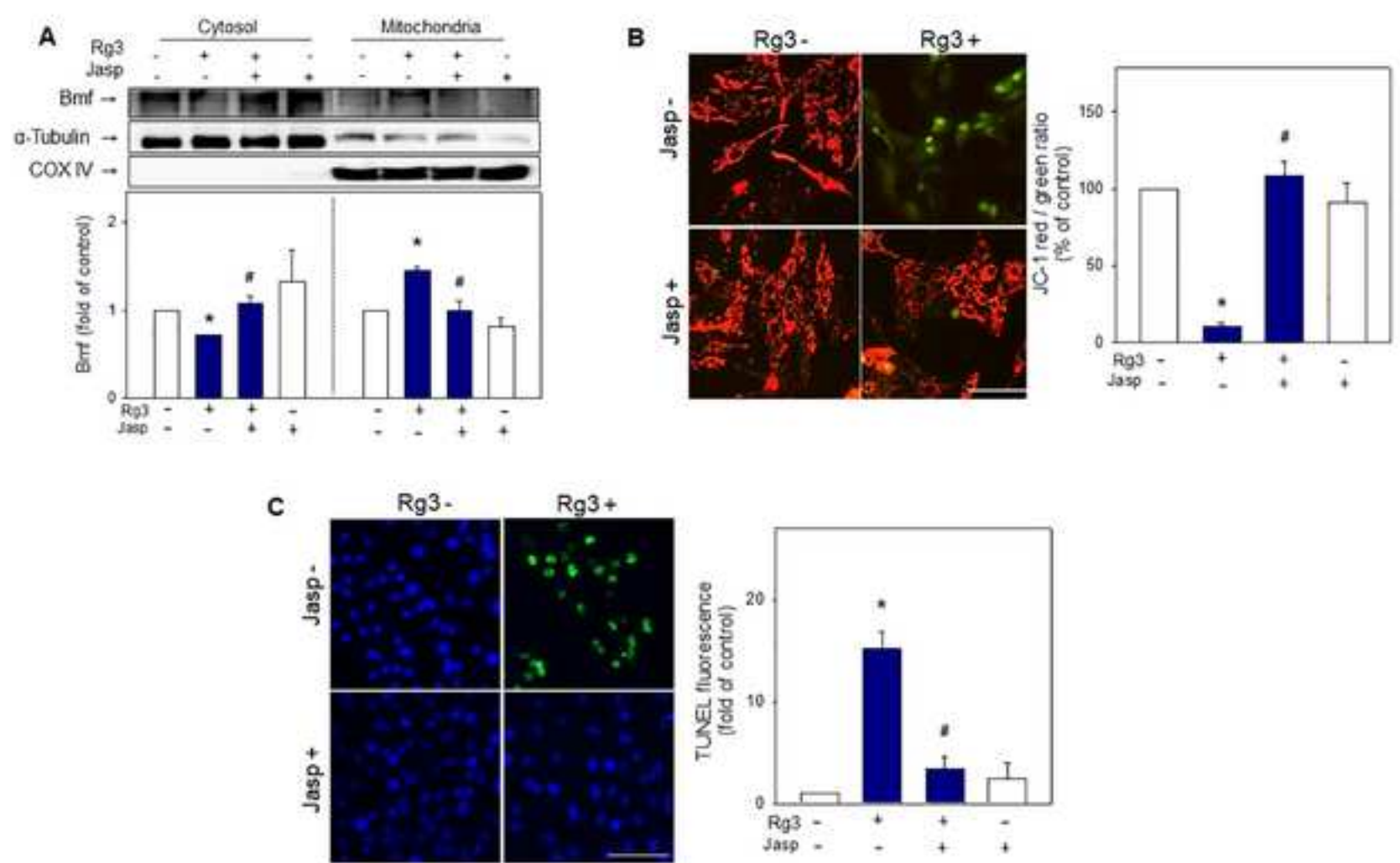


Figure 3

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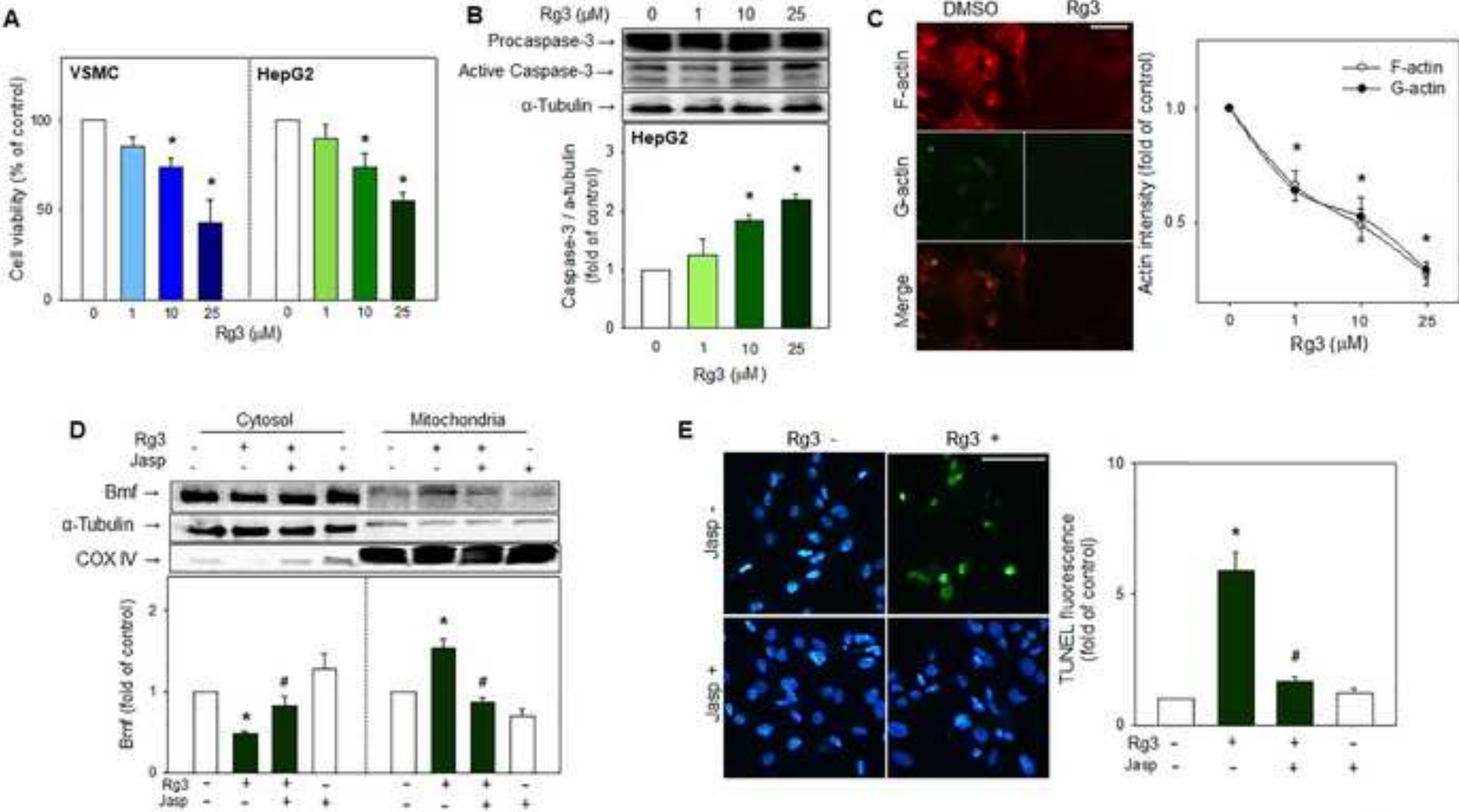
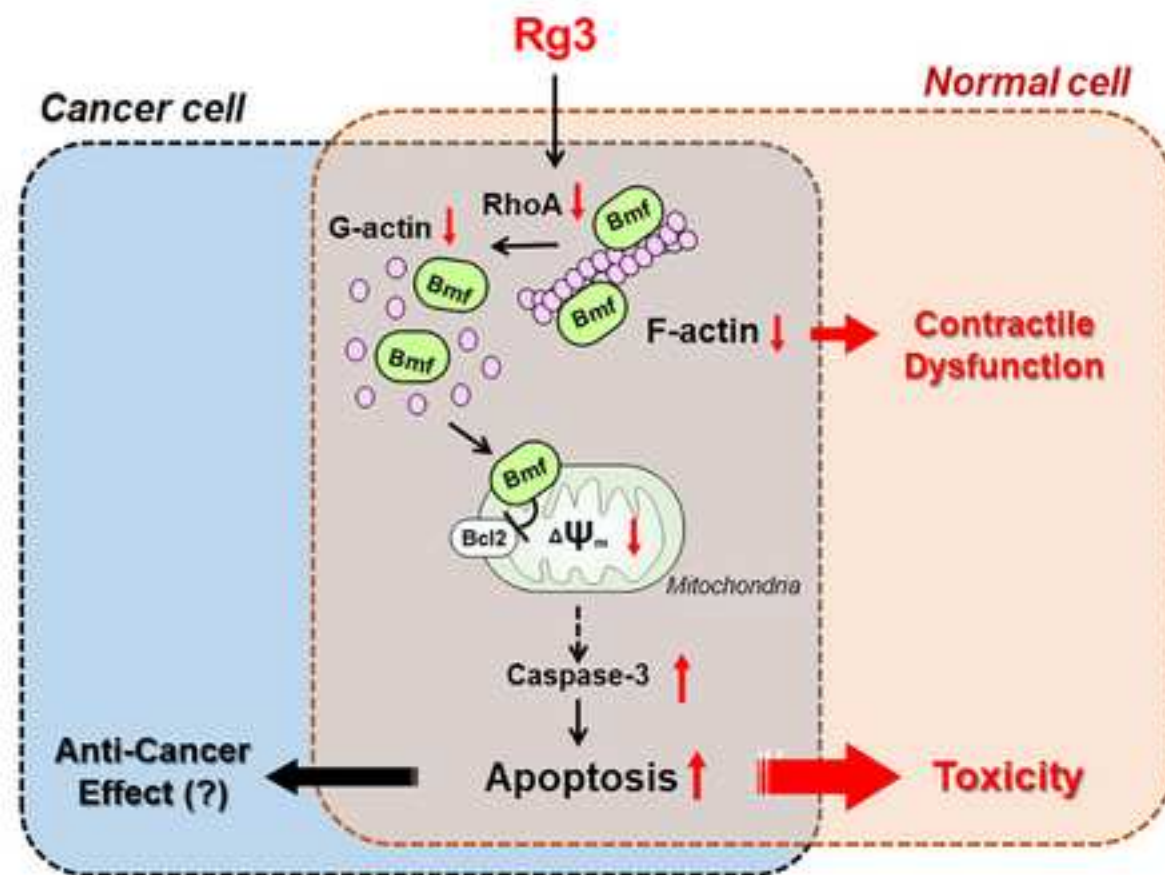


Figure 4



Scheme 1