Vascular Smooth Muscle Dysfunction and Remodeling Induced by Ginsenoside Rg3, a Bioactive Component of Ginseng

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Ginseng, one of the most well-known herbal medicines, is widely and indiscrimately used among the patients with cardiovascular disorders, raising concern over abuse of this medicine and unwanted effects. In this study, we investigated the effects of ginsenoside Rg3 (Rg3), an active ingredient of ginseng, on vascular contractility and structural integrity to explore its potential vascular toxicity. In isolated rat aorta, Rg3 suppressed the normal agonist-induced contractile response. This suppression persisted even after a rigorous washout. In the endothelium-denuded aortic ring, impairment of vascular contractility by Rg3 was retained, suggesting that vascular smooth muscle was affected. In primary vascular smooth muscle cells, Rg3 abolished agonist-induced Ca2+ increase, indicating that Ca2+ regulation was disrupted. Rg3 suppressed the contraction induced by Bay K8644, an L-type Ca2+ channel activator, whereas store-operated Ca2+ channel or intracellular Ca2+ store-mediated contraction was not affected, suggesting that the L-type Ca2+ channel was selectively impaired by Rg3. These in vitro results were further confirmed in vivo where Rg3 treatment significantly attenuated the agonist-induced pressor response. More importantly, 4-week repeated treatment with Rg3 in normal animals induced eutrophic remodeling in the thoracic aorta, that is, it brought about an increased luminal area without changes in the wall area. These results suggest that Rg3 can induce the vascular smooth muscle dysfunction by disturbing Ca2+ influx from the L-type Ca2+ channel, ultimately leading to impaired vascular contractility and structural remodeling.

Key Words: smooth muscle dysfunction; ginsenoside Rg3; extracellular Ca2+ influx.

Cardiovascular disease (CVD) is a leading health problem throughout the world, but because of a various disease etiology and frequent failures of conventional therapies, many patients often resort to complementary and alternative medicines (CAM). Approximately 40% of CVD patients in the United States are known to take herbal medicines, herbal nutritional supplements, or diet-based therapies as a type of independent or conjunctive treatment (Buettner et al., 2007; Nader et al., 2000; Yeh et al., 2006). Rising concern over the indiscrimuse of CAM has followed, however, with several reports warning of its potential adverse health effects (Ernst, 2007; Pharand et al., 2003).

Ginseng, one of the most well-known herbal medicines in the world, is consumed widely by CVD patients as a type of preventive or curative therapy, especially among Asian and Asian-American ethnic groups. Various ginseng products are marketed worldwide as a health food or dietary supplement. In China, an iv formula of ginseng is even offered for therapeutic use (Haijiang et al., 2003). In concert with this popularity, considerable effort in the scientific community is being made to elucidate the pharmacological effects of ginseng and its active ingredient, ginsenoside, on the cardiovascular (CV) function. In contrast, several cases of adverse effects of ginseng on the CV function have also been reported in relation to derailed blood pressure control (Siegel, 1979, 1980). According to the WHO database, hypertension and hypotension associated with a ginseng monopreparation have been frequently reported (Coon and Ernst, 2002; Ernst, 2002). However, few studies have focused on possible adverse effects on the CV function associated with ginseng consumption.

Ginsenosides Rg3 (Kim et al., 1999, 2003), Rb1, and Re (Scott et al., 2001) reportedly display antihypertensive and cardioprotective effects. Thus, they are regarded as active ingredients for the CV effects of ginseng. In particular, ginsenoside Rg3 has been studied extensively because of its potent effects on CV functions. The antihypertensive and cardioprotective effects of Rg3 were suggested to stem from the increased release of vasodilator molecule, nitric oxide (NO), and modulation of the Ca2+ ion channel (Kim et al., 1999, 2006). Most studies related to ginsenoside Rg3, however, were conducted under the premise that Rg3 has beneficial effects. Therefore, the effect of Rg3 treatment on the normal vascular
function or vascular structural integrity has not been properly addressed to the best of our knowledge.

Blood vessels, composed of endothelium and smooth muscle, maintain the balance of the vasomotor tone through contraction and relaxation (Larsen et al., 2006; Navarro-Antolin et al., 2005). Whereas endothelium controls the vasomotor tone by releasing the vasodilator NO and the vasoconstrictor endothelin, smooth muscle directly regulates the vasomotor tone through contraction and relaxation (Sanders, 2001). When a contractile agonist binds to a receptor, smooth muscle contraction is accomplished through two stages of tension generation and maintenance, that is, phasic and tonic tension (Morano, 2003; Shan et al., 1996). Phasic tension is initiated by a cytosolic Ca\(^{2+}\) increase from intracellular stores, a voltage-dependent Ca\(^{2+}\) channel such as the L-type Ca\(^{2+}\) channel or a store-operated Ca\(^{2+}\) channel (SOCC) such as the transient receptor potential channel C (TRPC) (Akata, 2007; Khalil, 2005). Tonic tension is maintained by regulating the dephosphorylation of the phosphomyosin light chain (Parekh and Putney, 2005; Taggart and Wray, 1998).

This contractile molecular machinery is elaborately controlled to maintain adequate hemodynamic homeostasis; however, it is vulnerable to chemical or pathological stress. Many chemicals can impair maintenance of the vasomotor tone, including milrinone, minoxidil, and arsenic (Bohr et al., 1991; Mohri et al., 1998; Partridge et al., 2005). These chemical stimuli affect Ca\(^{2+}\) channels, tension generation, or relaxation pathways (Bae et al., 2008a), causing irreversible alteration of vascular reactivity to contractile agonists, disturbing vascular compliance and hemodynamic homeostasis, and ultimately leading to permanent vascular dysfunction and structural vascular remodeling.

This study found that Rg3 irreversibly suppresses agonist-induced vasoconstriction through the disruption of Ca\(^{2+}\) homeostasis in vascular smooth muscle. We elucidate the underlying mechanism and examine the in vivo effect of single and repeated Rg3 treatment on blood pressure control and vascular structure in normal animals in an effort to understand the potential vascular toxicity of CAM.

**MATERIALS AND METHODS**

**Reagents.** The following chemicals were purchased from Sigma Chemical Co. (St Louis, MO): phenylephrine (PE), endothelin-1 (ET-1), serotonin creatinine sulfate, lucigenin, nicotineamide adenine dinucleotide, pyruvate, trichloroacetic acid, Triton X-100, N\(^{\text{O}}\)-nitroarginine, mandenione, bovine serum albumin, (−)-(S)-Bay K8644, urethane, and dimethyl sulfoxide (DMSO). Fluo-4/AM was obtained from Molecular Probes (Eugene, OR), and reagents and media used in cell culture were purchased from Gibco Co. (Carlsbad, CA). (S)-Ginsenoside Rg3 was received in the form of a kind gift from Dr J. H. Park (Seoul National University, Seoul, Korea). For the in vivo studies, ginsenoside Rg3 (1 g, purity > 98%) was purchased from Fleton Co., (Chengdu, China) through a custom order. Monomethylxanthine acid (MMA\(^{\text{II}}\)) was purchased from Dr Cullen at the University of British Columbia. All other reagents used were of the highest purity available.

**Animals.** Male Sprague-Dawley rats (Orient Co., Korea) weighing 250–350 g or male ICR mice (25–30 g) were used in all experiments. Before the experiments, the animals were acclimated for 1 week. Food and water were provided ad libitum. All protocols were approved by the Ethics Committee of the Animal Service Center at Seoul National University.

**Measurement of vasoconstriction in isolated aortic rings.** After the rats were humanely decapitated by exsanguination, the thoracic aorta was carefully isolated and cut into ring segments in lengths of 3–4 mm on ice. Aortic rings without endothelium were prepared by gently rubbing the intimal surface of the aortic rings with a cotton swab. The rings were then mounted on organ baths filled with Krebs-Ringer solution (KR solution; 115.5 mM NaCl, 4.6 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 2.5 mM CaCl\(_2\), 25.0 mM NaHCO\(_3\), and 11.1 mM glucose, pH 7.4) continuously saturated with a mixed gas of 95% O\(_2\) and 5% CO\(_2\) and maintained at 37°C. The change in tension was measured with a Grass FT03 force transducer (Grass Instrument Co., Quincy, MA) and was recorded using the AcqKnowledge III device (BIOPAC Systems Inc., Goleta, CA). To investigate the effect on vasoconstriction, the aortic rings were pretreated with Rg3 or vehicle (DMSO) and vasoconstriction was initiated by the cumulative addition of PE, serotonin, or ET-1. In order to examine whether Rg3 affects the stage of tension maintenance, that is, the tonic tension, Rg3 treatment continued for 10 min after the induction of vasoconstriction by PE.

**Measurement of cytosolic Ca\(^{2+}\) changes in primary rat smooth muscle cells.** The cytosolic Ca\(^{2+}\) level was measured in primary smooth muscle cells isolated from rat thoracic aorta based on a method described previously (Bkaily et al., 1999; Lee et al., 2006b). After the endothelium and adventitia were removed, aortic rings were chopped finely and smooth muscle cells were liberated from the tissue using collagenase and elastase (Worthington Biochemical Corp., Lakewood, NJ). After culturing at 37°C in a 95% air/5% CO\(_2\) incubator for 3 days with Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, smooth muscle cells were seeded on a chambered cover glass at 1 × 10\(^5\) cells/ml for 48 h and Rg3 or vehicle was treated for a further 16 h. Cells were then washed with a standard phosphate salt solution (140 mM NaCl, 5.0 mM KCl, 1.4 mM MgCl\(_2\), 2.0 mM NaH\(_2\)PO\(_4\), 10 mM NaHCO\(_3\), 4.2 mM glucose, pH 7.4) three times, and Fluo-4/AM (3 μM) was loaded for 60 min at room temperature in the dark. After the cells were rinsed, the cytosolic Ca\(^{2+}\) increase was initiated by the addition of PE (10 \(^{-5}\) M) under continuous measurement by a confocal microscope (Leica, Germany). The fluorescence intensity (488 nm excitation and 520–550 nm emission) was measured for the calcium signal, and the ratio of F (peak fluorescence intensity after PE addition) over F0 (fluorescence intensity at baseline) was calculated to indicate the cytosolic Ca\(^{2+}\) increase.

**Determination of Rg3 effects on intracellular and extracellular Ca\(^{2+}\) pathways.** To examine the effect of Rg3 on the contraction mediated by the release of the intracellular Ca\(^{2+}\) store, vasoconstriction was induced in a Ca\(^{2+}\)-free condition as previously described (Chew et al., 2003). After treatment with Rg3 or vehicle of the aortic rings without an endothelium for 10 min, the KR solution was replaced with a Ca\(^{2+}\)-free KR solution (120.0 mM NaCl, 5.0 mM KCl, 1.2 mM NaH\(_2\)PO\(_4\), 1.2 mM MgCl\(_2\), 25.0 mM NaHCO\(_3\), 2 mM ethylene glycol tetracetic acid, and 11.5 mM glucose, pH 7.4) and PE (10 \(^{-5}\) M) was added to initiate vasoconstriction. For selective activation of SOCC, the aortic rings without an endothelium were pretreated with 1 μM thapsigargin (an inhibitor of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase) for 90 min in Ca\(^{2+}\)-free KR solution to deplete the intracellular Ca\(^{2+}\) store. Aortic rings were then treated with Rg3 or vehicle for 10 min, and the bath solution was exchanged into KR solution containing 2.5 mM Ca\(^{2+}\) to induce SOCC-mediated contraction. TRPC inhibitors, 2-aminophenoxyethylphosphoryl borate (2-APB) and bromoethanol lactone (BEL), were used as positive controls. To investigate the effect of Rg3 on the contraction induced by the influx of extracellular Ca\(^{2+}\), Bay K8644, an L-type Ca\(^{2+}\) channel opener was used as previously described (Fusi et al., 2000). Bay K8644 was added cumulatively in 15 mM K\(^{+}\) buffer solution (105 mM NaCl, 15.0 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 2.5 mM CaCl\(_2\), 25.0 mM NaHCO\(_3\), 0.026 mM EDTA, and 11.1 mM glucose, pH 7.4) to initiate vasoconstriction.

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Measurement of L-type Ca\(^{2+}\) channel currents. To examine the direct effect of Rg3 on the L-type Ca\(^{2+}\) channel, the Ca\(^{2+}\) channel was expressed in *Xenopus* oocytes as previously described (Lee et al., 2006a). A single oocyte expressing the L-type Ca\(^{2+}\) channel was placed in a small Plexiglas net chamber. The microelectrodes were filled with 3M KCl, showing a resistance of 0.2–0.7 MΩ. Two-electrode voltage-clamp recordings were made at room temperature with an Oocyte Clamp (OC-725C, Warner Instrument) and a Digitada 1200A. The recording solution consisted of 10mM Ba(OH)\(_2\), 90mM NaOH, 2mM KOH, 5mM HEPES (pH 7.0 adjusted with methanesulfonic acid), and 0.3mM nifluamic acid.

Measurement of lactate dehydrogenase leakage and reactive oxygen species generation. To examine the nonspecific cytotoxicity of Rg3, the extent of lactate dehydrogenase (LDH) leakage from aortic rings without an endothelium was measured using a previously described method (Suenaga and Kamata, 1999). Generation of reactive oxygen species (ROS) was determined by lucigenin-enhanced chemiluminescence (Lee et al., 2001). Aortic rings were placed into tubes filled with KR solution containing 0.25mM lucigenin and various concentrations of Rg3. Luciferin-enhanced chemiluminescence was measured at 60 min using a luminometer (Berthold, Germany).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining of aortic rings and histopathological assessment. Aortic rings were placed in minimum essential media containing 100 U/ml penicillin and 100 μg/ml streptomycin, and Rg3, niflumic acid, MMA\(_{10}\), or vehicle was used as a treatment. They were then incubated in a 95% air/5% CO\(_2\) incubator for 18 h at 37°C. After incubation, the aortic rings were fixed in a buffered formalin solution (10%) and embedded in paraffin. Terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assays were done using a commercial kit according to the manufacturer’s instructions (Chemicon International, Temecula, CA). The embedded tissue was sectioned at a thickness of 4 μm and was placed on an adhesive slide. The sections were deparaffinized by washing with xylene following serial dehydration with ethanol (100, 95, 80, and 70%). Dehydrated sections were treated with 0.3% H\(_2\)O\(_2\) to quench endogenous peroxidase activity followed by 20 μg/ml DNase-free Proteinase K to retrieve antigenic epitopes. Subsequently, the sections were treated with TdT enzyme reagent for 1 h at 37°C to label-free 3'-OH termini with digoxigenin-dUTP. To detect incorporated digoxigenin–conjugated nucleotides, horseradish peroxidase–conjugated anti-digoxigenin antibody and 3,3'-diaminobenzidine (DAB) were used. Sections were treated with anti-digoxigenin-peroxidase for 30 min at room temperature, and this was followed by DAB development. The sections were counterstained with Mayer’s hematoxylin. Dehydrated sections were then cleaned in xylene and mounted. For assessment of TUNEL-positive cells, the numbers of total cell nuclei and positive cell nuclei were counted in four fields for each specimen and the percentages of positive cell nuclei were calculated.

Measurement of the blood pressure. After anesthetizing the rats with urethane (1250 mg/kg, ip), the left carotid artery was surgically cannulated with a polyethylene tube filled with heparinized saline (20 U/ml) for the measurement of the blood pressure; the left jugular vein was used for the iv injection. The arterial pressure was monitored with an MLT 0380 transducer and Power Lab data acquisition system interfaced to Chart 5 software (ADI Instruments, Australia). After Rg3 (10 mg/kg/min) or vehicle (5% DMSO in saline) was administered by iv infusion for 2 min (total administered Rg3 was 20 mg/kg in 10 ml/kg) to minimize the procedural impact on the blood pressure measurement, the PE-elevated mean arterial pressure was measured to evaluate the effects of Rg3 on the agonist-induced pressor response.

Evaluation of vascular remodeling by repeated treatment of Rg3. Rg3 (20 mg/5 ml/kg) or vehicle (10% DMSO in saline) was administered by iv bolus to male ICR mice once daily for 4 weeks (five times a week), and the animals then were humanely sacrificed to obtain the ascending thoracic aorta through formalin perfusion. Thoracic aorta were fixed in a buffered formalin solution (10%) and embedded in paraffin. Horizontal ring sections with a thickness of 3 μm were carefully obtained without folds at identical points in the aorta, and hematoxylin and eosin staining was used for microscopic observation. The area of the lumen and the wall thickness were measured and analyzed from digitalized microphotographs using the commercial image analyzer software, Image pro Plus (Media Cybernetics, Bethesda, MD). For the estimation of the luminal area, the circumference of the lumen was obtained and used to calculate the area assuming a circular structure, according to the method described previously (Harmon et al., 2000). For the wall thickness, eight points around the circumference were randomly measured and averaged. The wall area was calculated from the wall thickness and the circumference of the lumen.

Statistical analysis. All the data are represented by mean ± SEM, and the data were subjected to one-way analysis of variance followed by Duncan’s multiple ranged tests to determine which means were significantly different from the control. Statistical analysis was performed using SPSS software (Chicago, IL). In all cases, p value of < 0.05 was used to determine significance.

RESULTS

To investigate the effect of Rg3 on blood vessel contraction, freshly isolated rat aortic rings were pre-incubated with Rg3 and PE, a typical contractile agonist, was added cumulatively to initiate vasoconstriction. Although Rg3 alone did not affect the vasomotor tone (data not shown), Rg3 attenuated the PE-induced contraction in a concentration- and time-dependent manner (Figs. 1A and 1B). The contractions induced by serotonin and ET-1, other widely used contractile agonists, were also suppressed by Rg3 (Fig. 1C), and the decreased contraction by Rg3 was not recovered even after the Rg3 was rinsed off thoroughly (Fig. 1D), suggesting that Rg3 caused nonspecific and irreversible suppression of the normal vascular contractility.

To examine the mechanism underlying the Rg3-induced contractile dysfunction, aortic rings without endothelium were employed to determine which component of the vessel, that is, the endothelium or smooth muscle, is affected by Rg3. As shown in Figure 2A, the inhibitory effect of Rg3 on PE-induced contraction was retained in the endothelium-denuded aortic rings, implying that Rg3 disrupts the contractile function of smooth muscle directly. This effect was not reversed even after the removal of Rg3 (Fig. 2B), in good accordance with the result obtained with endothelium intact rings (Fig. 1D). Generally, the contraction of smooth muscle develops via the process of phasic tension, the initiation of contraction, followed by tonic tension, the maintenance of contraction (Morano, 2003). The posttreatment of Rg3 on aortic rings without an endothelium after the induction of vasoconstriction had no effect, whereas the pretreatment inhibited the PE-generated tension significantly (Figs. 2A and 2C), reflecting that only the phasic tension was affected by the Rg3 treatment. The effect of Rg3 on agonist-induced vasoconstriction is depicted in Figure 2D.

Tension generation was mainly mediated by agonist-induced cytosolic Ca\(^{2+}\) elevation (Akata, 2007). The effect of Rg3 on Ca\(^{2+}\) mobilization was examined in fluorescent Ca\(^{2+}\) dye fluo-4-loaded primary smooth muscle cell isolated from rat thoracic aorta using confocal microscopy. As shown in Figure 3A, PE-induced cytosolic Ca\(^{2+}\) increase was completely blocked by
Rg3, indicating that Rg3 disturbed the agonist-initiated Ca\textsuperscript{2+} mobilization step. The agonist-induced cytosolic Ca\textsuperscript{2+} increase stemmed either from the release of intracellular Ca\textsuperscript{2+} stores or from the influx of extracellular Ca\textsuperscript{2+} through the voltage-sensitive L-type Ca\textsuperscript{2+} channel or the SOCC such as TRPC (Akata, 2007). First, to examine the effect of Rg3 on the release from intracellular Ca\textsuperscript{2+} stores, a Ca\textsuperscript{2+}-free buffer system was employed in which agonist-induced contraction depends wholly on the release from the intracellular Ca\textsuperscript{2+} store. Rg3 did not inhibit PE-induced smooth muscle contraction in the Ca\textsuperscript{2+}-free buffer system (Fig. 3B, left side), reflecting that its mechanism was not related to the intracellular Ca\textsuperscript{2+} stores.

Second, to investigate the effect of Rg3 on the extracellular Ca\textsuperscript{2+} influx from SOCC, the intracellular Ca\textsuperscript{2+} store was depleted by a selective sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase inhibitor, thapsigargin, in a Ca\textsuperscript{2+}-free buffer system, and SOCC-mediated vasoconstriction was induced with an addition of extracellular Ca\textsuperscript{2+}. As shown in Figure 3B (right side), contraction mediated by SOCC was not affected by Rg3, whereas significant inhibition was accomplished by the positive TRPC inhibitors, 2-APB and BEL. To investigate the effect of Rg3 on the L-type Ca\textsuperscript{2+} channel, a major voltage-sensitive Ca\textsuperscript{2+} channel in vascular smooth muscle, Bay K8644, the activator of the L-type Ca\textsuperscript{2+} channel, was used (Wamhoff et al., 2004).
As opposed to other Ca\(^{2+}\) channels, Rg3 inhibited Bay K8644-induced vasoconstriction in a concentration-dependent manner (Fig. 3C), indicating that Rg3 altered the Ca\(^{2+}\) elevating pathway by blocking the L-type Ca\(^{2+}\) channel. A direct effect of Rg3 on L-type Ca\(^{2+}\) channel could be further confirmed in Xenopus oocytes expressing the L-type Ca\(^{2+}\) channel with a patch-clamp assay (Fig. 3D). Similar to Figures 1D and 2B, the inhibitory effect of Rg3 on Bay K8644-induced contraction and on the Ca\(^{2+}\) channel was not restored even after the removal of Rg3 (Figs. 3C and 3D).

To investigate the long-term effect of Rg3 exposure on vascular smooth muscle, PE-induced vasoconstriction was measured in the presence of Rg3 or 1 and 4 h after the removal of Rg3. Even after a longer washout and recovery time, the effect of Rg3 was retained (Fig. 4A), which was similar to MMA\(^{III}\), organic arsenical associated with various CVDs (Yoshida et al., 2004) (Fig. 4B). These irreversible effects of Rg3 and MMA\(^{III}\) showed a good contrast to the reversible effect of nifedipine, a widely used antihypertensive and L-type Ca\(^{2+}\) channel blocker (Fig. 4C), suggesting that an Rg3-induced persistent effect can be regarded as a dysfunction rather than as a type of pharmacological modulation. To investigate if nonspecific cytotoxicity is involved in Rg3-mediated smooth muscle dysfunction, we measured the leakage of LDH, as a marker for membrane disturbance (Bae et al., 2008b; Shukla et al., 2001), in endothelium-denuded aortic rings after incubation with Rg3. Rg3 did not induce LDH release (Fig. 4D), showing that Rg3 does not induce nonspecific cytotoxicity. Alteration of the vascular tone can be induced by ROS generation or inducible NO synthase (iNOS) induction (Ardanaz and Pagano, 2006; Lyle and Griendling, 2006). As shown in Figures 4E and 4F, however, ROS generation or iNOS was not involved in Rg3-induced vascular dysfunction. Interestingly, it was also found that long-term incubation (18 h) of aortic rings with Rg3 and MMA\(^{III}\) induced apoptosis of smooth muscle cells, whereas nifedipine did not (Fig. 4G).

To investigate the in vivo consequences of these effects on smooth muscle contraction, the effect of Rg3 on PE-induced blood pressure elevation was measured. Without any alteration of the heart rate (data not shown), the Rg3-treated group showed attenuation of the PE-induced pressor response (Fig. 5A), suggesting that normal vascular smooth muscle contractility was impaired by Rg3 in vivo. Of particular note, subchronic administration of Rg3 (20 mg/kg iv for 4 weeks) to normal animals resulted in a distended thoracic aorta, as
determined by increased lumen and decreased arterial wall thickness (Fig. 5B) without a significant increase in the medial area (5035 ± 2475 μm² in control, N = 7 vs. 4558 ± 1337 in Rg3, N = 8, p = 0.103), indicating that repeated exposure to Rg3 can indeed bring about permanent changes in the vascular structure.

DISCUSSION

This study demonstrated that Rg3, an active ginsenoside in ginseng, can induce smooth muscle dysfunction through the irreversible attenuation of extracellular Ca²⁺ influx via the L-type Ca²⁺ channel. These effects were different from the therapeutic effects of the antihypertensive drug, nifedipine, but rather similar to the vascular toxicant, MMAIII, in that they were irreversible even after a long washout period. Of particular note, Rg3 impaired the normal PE-induced pressor response and after long-term exposure induced vascular remodeling in thoracic aorta as shown by the increased luminal area and decreased wall thickness without changes in the wall area. This indicates that Rg3 can induce smooth muscle dysfunction and permanent structural changes. Through this study, we believe that important evidence is provided regarding potential vascular dysfunction and CV toxicity by CAM.

Unlike newly synthesized chemical drugs, herbal medicines used as CAM are generally regarded with a positive attitude by researchers, government regulators, and consumers. Frequently, they are taken by general consumers without appropriate safety evaluations (Ang-Lee et al., 2001). In particular, ginseng and ginsenoside-enriched products created through steaming have been marketed worldwide as diverse consumer products such as functional foods, beverages, and dietary supplements. Recent reports on the cardioprotective and antihypertensive effects of ginsenosides and ginseng have further added to the enthusiasm for ginseng products, exposing the general population to an extremely high level of ginseng and its ingredients. As seen in the present investigation, the dysfunction of vascular smooth muscle through the irreversible suppression of the L-type Ca²⁺ channel by Rg3 may underlie an antihypertensive or a cardiac effect of ginseng. In this regard, we suggest that the CV effects of herbal medicines or CAM, including ginseng-processed products, should be reviewed carefully to discern their possible involvement of smooth muscle dysfunction.

FIG. 3. Effect of Rg3 on Ca²⁺ mobilization and vasoconstriction. (A) Inhibition of PE (10⁻⁵ M)-induced calcium increase by Rg3 (25 μM, 10 min) was observed in primary smooth muscle cell using fluo-4/AM in confocal microscopy. Scale bar, 20 μm. Representative images and the increase in the fluorescence (F) induced by PE were expressed as the ratio of basal fluorescence (F₀). (B) Effect of Rg3 (25 μM, 10 min) on PE (10⁻⁵ M)-induced vasoconstriction in a Ca²⁺-free buffer condition (left) and SOCC-mediated vasoconstriction in aortic rings (right). (C) After Rg3 (25 μM) was treated to aortic rings without an endothelium for 10 min, vasoconstriction was initiated with an addition of Bay K8644, an L-type Ca²⁺ channel opener. The irreversible effect of Rg3 on Bay K8644-induced contraction was measured 1 h after rinsing out the Rg3-containing KR buffer in an organ bath. The Bay K8644 (10⁻⁷ M)-induced contraction at point ‘’I’’ was measured in an Rg3-containing KR buffer, whereas that of ‘’II’’ was measured after rinsing out. (D) The inset shows the representative current traces obtained from oocytes as a control and after 10 min of application of 25 μM Rg3. The current-voltage relationships of the L-type Ca²⁺ channels were obtained in the absence or presence of 25 μM Rg3. Values are means ± SEMs of three to four independent experiments. * Represents significant differences from the controls (p < 0.05).
Previously, most studies centering on Rg3 were conducted on the premise that the effects of Rg3 stemmed from the pharmacological modulation of biological targets including Ca$^{2+}$ channels and iNOS (Kim et al., 1999; 2006). In particular, the modulation of voltage-sensitive Ca$^{2+}$ channels was suggested as a key mechanism for the actions of Rg3 and other ginsenosides (Bai et al., 2004; Choi et al., 2001; Jeong et al., 2004). These studies, however, employed relatively short
incubation times of 1 or 5 min and focused mainly on the immediate action of ginsenosides on ion channels, thus leaving open the possibility of overlooking potential adverse effects after long-term exposure to ginsenosides. As shown in the aortic ring system as investigated in this study, with primary smooth muscle cells and the L-type $\text{Ca}^{2+}$ channel expressing *Xenopus* oocytes, prolonged exposure to Rg3 brought about irreversible and selective suppression of L-type $\text{Ca}^{2+}$ channel–mediated contraction, indicating that $\text{Ca}^{2+}$ modulation by Rg3 may in fact stem from the impairment of the L-type $\text{Ca}^{2+}$ channel and smooth muscle dysfunction.

Examples of chemical-induced vascular dysfunction can easily be found with various types of chemicals including drugs. Vascular dysfunction can impair the normal vascular contractile response, ultimately leading to vascular injury in vivo (Kerns et al., 2005). The derangement of vascular reactivity is widely recognized as a major complication in diseases associated with peripheral or central circulatory problems such as diabetes, sepsis, cirrhosis, and hyperthyroidism (McAllister et al., 1998; Nobe et al., 2004; Schepke et al., 2001). As adequate pressor stimulation is essential in arterial compliance and for the maintenance of the blood flow to vital organs, dysfunction of the vascular contractile response is often related to abnormal hemodynamic conditions, insufficient blood perfusion, vascular lesions, and tissue damage in these diseases. In this regard, the possibility that long-term and chronic exposure to Rg3 induces or complicates CVD cannot be excluded completely. In support of this, smooth muscle dysfunction by Rg3 was somewhat similar to the effects of a vascular toxicant, MMA$^\text{III}$, which irreversibly and nonspecifically impairs agonist-induced smooth muscle contraction through the disruption of calcium mobilization (Bae et al., 2008a). In addition, the present study showed that long-term incubation with Rg3 and MMA$^\text{III}$ induced apoptosis of smooth muscle cells, whereas nifedipine did not (Fig. 4G). MMA$^\text{III}$, a methylated metabolite of arsenical, has been suggested as an important toxic metabolite for arsenic-related CVDs such as black foot disease, peripheral artery disease, and aneurysm (Engel and Smith, 1994; Yoshida et al., 2004), suggesting that Rg3 induces MMA$^\text{III}$-like CV toxic effects and ultimately may therefore be related to vascular diseases.

It is conspicuous that repeated administration of Rg3 in vivo resulted in an increased luminal area in arteries. It appears that chronic impairment of an adequate pressor response and smooth muscle dysfunction as shown in vitro resulted in permanent changes in the vascular structure in vivo. The plasma concentration of Rg3 after iv dosing at 20 mg/kg is not
known, but Xie et al. (2005) reported that the plasma concentration of Rg3 30 min after iv administration at 1 mg/kg in rats was approximately 1 µg/ml. Based upon this report, the plasma concentration of Rg3 at 30 min after 20 mg/kg iv administration can be estimated roughly to be 20 µg/ml (−25µM), which is similar to the Rg3 concentration adopted in our in vitro studies further supporting our conclusion. Despite the well-correlated in vitro and in vivo evidence provided by the present study, the actual consequence of Rg3-induced smooth muscle dysfunction in humans warrants further studies. Although some cases of iv dosing of ginsenosides indeed exist, oral and chronic ingestion is the common route of exposure to Rg3 in humans. To simulate an actual oral ingestion scenario, lifetime chronic exposure studies would be required, which is beyond the scope of the present study. However, in our study, we would like to point out that Rg3 induced smooth muscle dysfunction after a relatively short exposure timeframe, suggesting that it may occur in real-life conditions in which humans are chronically exposed to Rg3 through lifetime consumption of ginseng-processed functional food or CAM.

Subchronic exposure to Rg3 resulted in an increased luminal area of the thoracic aorta, whereas an estimation of the wall area indicated no statistically significant change, suggesting that Rg3 induced a pattern of eutrophic outward remodeling, that is, an increased luminal area without a change in the wall area (Ward et al., 2000). This vascular remodeling may be a consequence of the adaptation of blood vessels to smooth muscle dysfunction and an impaired pressor response. An example of pathological eutrophic outward remodeling was reported by Crijns et al. (1999) in the mesenteric arteries of diabetic rat model. In their report, they suspected that the hemodynamic and subsequent biochemical changes in blood vessels from diabetes may have caused the eutrophic outward remodeling, suggesting that Rg3-mediated smooth muscle dysfunction and impaired vasomotor tone induced abnormal hemodynamic and biochemical changes in the thoracic aorta. These results are also well in line with the suggestion of De Mey et al. (2005) who held that long-term vasodilatation and flow increase can lead to outward remodeling through shifting the load borne by the contractile apparatus to the cytoskeleton and focal adhesion sites of the arterial smooth muscle cells. At this time without further details of biomarker expressions and other supportive data to explain the underlying mechanism, however, it remains unclear what implications these Rg3-induced structural changes may ultimately have on general CV homeostasis. Further studies should be conducted to elucidate the long-term consequences and the link with ginseng-related adverse effects.

In conclusion, this study demonstrates that Rg3 can induce irreversible impairment of agonist-induced vascular contraction through the blockade of Ca\(^{2+}\) mobilization via the L-type Ca\(^{2+}\) channel. More importantly, Rg3-induced vascular dysfunction was represented in vivo as an abnormal pressor response and as vascular remodeling. With this study, we believe that strong evidence is provided regarding the potential of CAM-induced vascular dysfunction and CV risk.

REFERENCES


