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Korean Red Ginseng protects endothelial cells from serum-deprived apoptosis by regulating Bcl-2 family protein dynamics and caspase *S*-nitrosylation

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Korean Red Ginseng extract (KRGE) is a traditional herbal medicine utilized to prevent endothelium dysfunction in the cardiovascular system; however, its underlying mechanism has not been clearly elucidated. We here examined the pharmacological effect and molecular mechanism of KRGE on apoptosis of human umbilical vein endothelial cells (HUVECs) in a serum-deprived apoptosis model. KRGE protected HUVECs from serum-deprived apoptosis by inhibiting mitochondrial cytochrome *c* release and caspase-9/-3 activation. This protective effect was significantly higher than that of American ginseng extract. KRGE treatment increased antiapoptotic Bcl-2 and Bcl-X$_L$ protein expression and Akt-dependent Bad phosphorylation. Moreover, KRGE prevented serum deprivation-induced subcellular redistribution of these proteins between the mitochondrion and the cytosol, resulting in suppression of mitochondrial cytochrome *c* release. In addition, KRGE increased nitric oxide (NO) production via Akt-dependent activation of endothelial NO synthase (eNOS), as well as inhibited caspase-9/-3 activities. These increases were reversed by co-treatment of cells with inhibitors of eNOS and phosphoinositide 3-kinase (PI3K) and pre-incubation of cell lysates in dithiothreitol, indicating KRGE induces NO-mediated caspase modification. Indeed, KRGE inhibited caspase-3 activity via *S*-nitrosylation. These findings suggest that KRGE prevents serum deprivation-induced HUVEC apoptosis via increased Bcl-2 and Bcl-X$_L$ protein expression, PI3K/Akt-dependent Bad phosphorylation, and eNOS/NO-mediated *S*-nitrosylation of caspases. The cytoprotective property of KRGE may be valuable for developing new pharmaceutical means that limit endothelial cell death induced during the pathogenesis of vascular diseases.

Keywords: *Panax ginseng*, Endothelial cells, Apoptosis, Bcl-2 family, Nitric oxide

INTRODUCTION

Endothelial cells, which constitute the inner cellular lining of blood vessels, have a key role in regulating vascular homeostasis and function, such as vascular integrity, vasorelaxation, and local inflammation [1]. Dysfunc-

tion of endothelial cells is associated with the pathogenic processes of a number of cardiovascular diseases, such as atherosclerosis, hypertension, heart attack, and stroke [1-3]. Although endothelial cell dysfunction occurs in many different disease processes, caspase-dependent apoptotic cell death induced by the extrinsic or intrinsic pathways is identified as a common denominator [4].

Endothelial cell apoptosis is regulated by cellular levels of the proapoptotic Bcl-2 family, cytosolic cytchrome *c*-dependent caspase activation, and activation of survival signals, including Akt [4,5]. Therefore, regulation of pro-apoptotic and antiapoptotic gene expression, caspase activation, and apoptotic signaling prevents endothelial cell apoptosis and maintains vascular function and integrity. Indeed, many risk factors for atherosclerosis cause endothelial cell apoptosis, and interventive treatments of atherosclerosis or its complications can reduce endothelial cell apoptosis [6]. Therefore, a protective strategy for endothelial cell apoptosis provides a great opportunity for the treatment of cardiovascular diseases.

Nitric oxide (NO) synthesized from L-arginine by NO synthases plays dichotomous regulatory roles under physiological and pathological conditions [7]. NO can promote apoptosis in some cells, whereas it inhibits apoptosis in other cell types, including endothelial cells [7]. Although NO produced by endothelial NO synthase (eNOS) has multiple beneficial effects on vascular function [7], our previous study demonstrated that NO can rescue endothelial cells from apoptosis by inactivating caspase-3 via *S*-nitrosylation [8]. Thus, decreased NO production in endothelial cells can promote endothelial apoptosis [9]. These evidences indicate that NO is a key molecule in preventing endothelial dysfunction, which is associated with vascular diseases.

Panax ginseng Meyer is well known as an oriental herbal medicine and is now extensively consumed as a medicinal product in the world. Among many ginseng products, Korean Red Ginseng possesses multiple biological activities, including prevention of endothelial dysfunction in hypertensive patients [10]. Our previous study showed that Korean Red Ginseng extract (KRGE) promoted endothelial cell function and angiogenesis, leading to the formation of a new vascular network [11]. However, the regulatory effects of KRGE on endothelial cell apoptosis have not been clearly elucidated. We here examined the biological and pharmacological effects of KRGE on endothelial cell apoptosis and its underlying mechanism in a serum-deprived apoptosis model.

We found that KRGE possesses antiapoptotic activity in serum-deprived endothelial cells by up-regulating antiapoptotic Bcl-2 and Bcl-X_L protein expression and activating phosphoinositide 3-kinase (PI3K)/Akt-dependent eNOS activation and Bad phosphorylation. Thus, our results suggest that Korean Red Ginseng may be considered as a potential therapeutic medicine for cardiovascular diseases induced by endothelial apoptosis and dysfunction.

MATERIALS AND METHODS

Materials

Culture medium and supplements were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Wortmannin and N-monomethyl-L-arginine (NMA) were obtained from Calbiochem (San Diego, CA, USA). The peptide substrates and caspase inhibitors were obtained from Alexis Corporation (San Diego, CA, USA). Antibodies used in this study were obtained from BD Biosciences (San Diego, CA, USA), Transduction Laboratories (Lexington, KY, USA), and Santa Cruz Biotechnology (Santa Cruz, CA, USA). 4-Amino-5-methylamino-2`7`-difluorofluorescein diacetate (DAF-FM diacetate) was purchased from Molecular Probes (Eugene, OR, USA). KRGE and American ginseng extract (AGE) were obtained from Korea Ginseng Corporation (Daejeon, Korea) and Wuhan Hezhong Bio-Chemical Manufature (Wuhan, China), respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise.

Cell viability assay

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as previously described [11] and used in passages 2-7. Cell viability was determined by using a Cell Counting Kit 8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). HUVECs (1×10^5 cells/well) were plated onto six-well plates in 1 mL of M199 containing 20% fetal bovine serum (FBS). Next day, the cells were switched to serum-free M199 with the indicated concentrations of KRGE or 5% serum-containing M199 as a control. After 48 h, cells were further incubated with CCK-8 solution for 4 h. Cell viability was determined by measuring absorbance at 450 nm using a microplate reader. Viability of control cells cultured in M199 with 5% FBS was set at 100% and viability relative to the control was presented. HeLa cells were cultured with 10 μg/mL of cisplatin (Sigma-Aldrich) alone or in combination with KRGE in Dulbecco's modified Eagle's medium containing 5% FBS for 24 h. Cell viability was deter-

mined by CCK-8 assay.

Caspase activity assay

Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4) containing a protease inhibitor mixture (5 mg/mL aprotinin and pepstatin, 10 mg/mL leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride). The cell suspension was lysed by three freeze-thaw cycles, and the cytosolic fraction was obtained by centrifugation at 12,000 xg for 10 min at 4°C. Caspase activities were determined by measuring proteolytic cleavage of the chromogenic substrates Ac-DEVD-pNA (caspase 3-like activity), Ac-LEHD-pNA (caspase 9-like activity), or Ac-IETD-pNA (caspase 8-like activity) as described previously [8]. In some experiments, cell lysates were preincubated with 20 mM dithiothreitol (DTT) on ice for 30 min before the caspase activity assay.

Western blotting and nitric oxide measurement

HUVECs were harvested and washed twice with ice-cold PBS, with protease inhibitors. Cell lysates were obtained by centrifugation at 12,000 x*g* at 4°C for 10 min following three cycles of freeze/thaw, and cytosolic and mitochondrial fractions were prepared as previously described [8]. Target protein levels were determined by Western blot analysis [8,11]. For measurement of intracellular NO levels, cells were treated with KRGE for 1 h and washed twice with serum-free medium and then incubated with 5 μM DAF-FM diacetate for 1 h at 37°C. After the excess probe was removed, the fluorescence intensity of the NO adduct of DAF-FM was captured from at least 10 randomly selected cells per dish using a confocal laser microscope [12].

Biotin-switch assay

S-nitrosylated caspase-3 was detected using biotin-switch assay system with some modifications from a previous method [13]. HUVECs were washed two times with cold phosphate-buffered saline and lysates were prepared by incubation with HEN buffer containing 250 mM HEPES, 1 mM ethylenediaminetetraacetic acid, 0.1 mM neocuproine (pH 7.7), sodium dodecyl sulfate (SDS) (2.5% final concentration), and methylmethane thiosulfonate (Sigma-Aldrich) at 50°C for 20 min. Proteins were precipitated with acetone, washed three times with 70% acetone, and mixed with 0.2 mM *N*-(6-(biotinamido) hexyl)-3′-(2′-pyridyldithio) propionamide (HPDP-biotin; Pierce, Rockford, IL, USA) with or without 50 mM

ascorbate at ambient temperature for 1 h. Biotinylated proteins were purified using streptavidin-agarose beads (Sigma-Aldrich), separated by SDS-polyacryl amide gel electrophoresis, and immunoblotted with anti-caspase-3 antibody.

Statistical analysis

All data were analyzed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA) and expressed as mean±SD of at least three separate experiments. A paired Student's t-test or one-way analysis of variance was used to assess significant differences. A value of $p<0.05$ was accepted as significant.
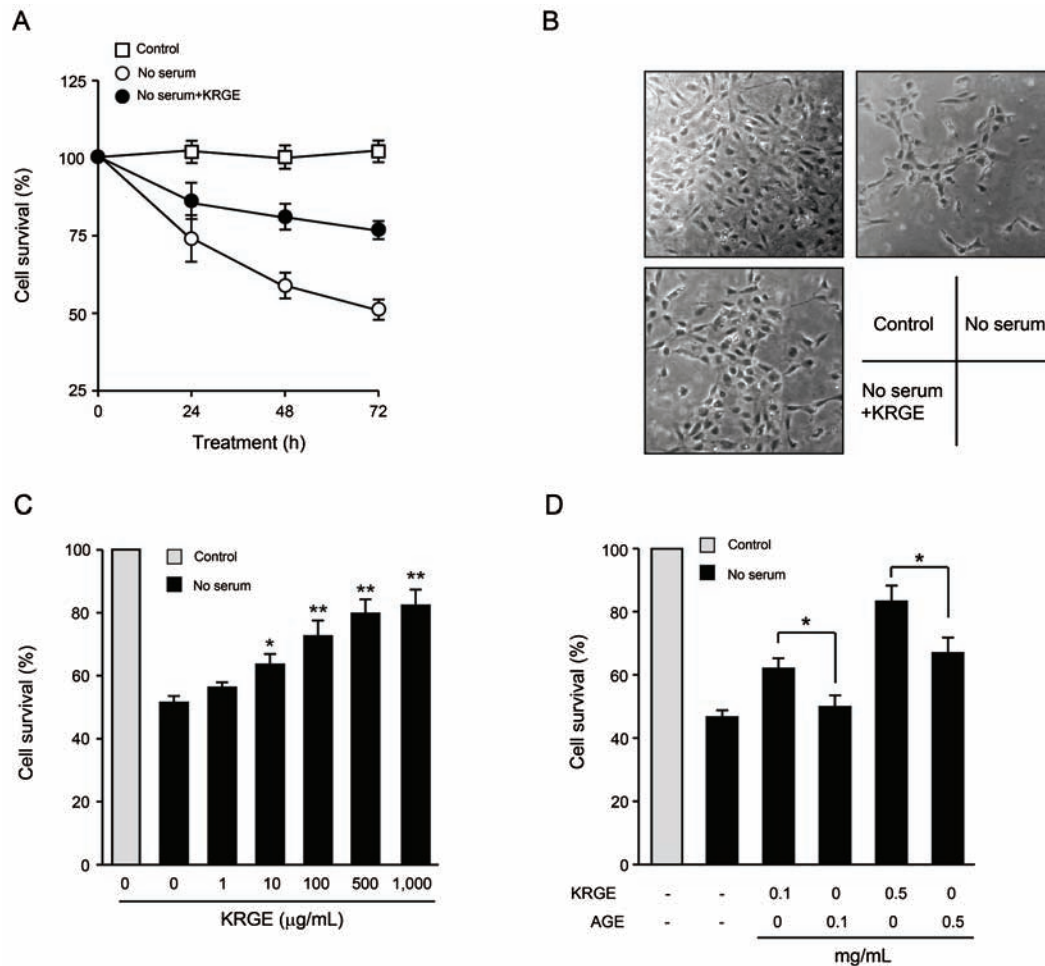
RESULTS

KRGE protects HUVECs from serum deprivation-induced cell death

We first examined whether KRGE protects HUVECs from serum deprivation-induced cell death. Serum deprivation significantly decreased endothelial cell survival in a time-dependent manner, with about 50% cell death at 72 h, as compared with control cells. However, this effect was inhibited when cells were treated with 0.5 mg/mL of KRGE (Fig. 1A). The protective effect of KRGE on serum deprivation-induced cell death was further confirmed by microscopic assay (Fig. 1B). KRGE also elicited a protective effect on serum deprivation-induced cell death in a dose-dependent manner (Fig. 1C). Interestingly, the protective effect of KRGE was significantly higher than that of AGE (Fig. 1D). These results suggest that KRGE prevents human endothelial cells from serum deprivation-induced cell death, with a significantly greater effect than AGE.

KRGE does not prevent tumor cell death

Since KRGE demonstrated a protective effect on serum deprivation-induced cell death in primary cultured human endothelial cells (Fig. 1), we next examined whether this extract regulates tumor cell death under the same experimental condition. Serum deprivation significantly decreased survival of the mouse melanoma cell line B16F1 and human cervical cancer cell line HeLa, and these effects were not altered by KRGE treatment (Fig. 2A, B). Furthermore, in anticancer drug cisplatin-induced HeLa cells, KRGE was observed to increase cell death rather than to serve a protective role (Fig. 2C). These results suggest that KRGE exerts a distinct survival effect with regard to serum deprivation-induced cell death in human normal endothelial cells, but not in tumor cells.
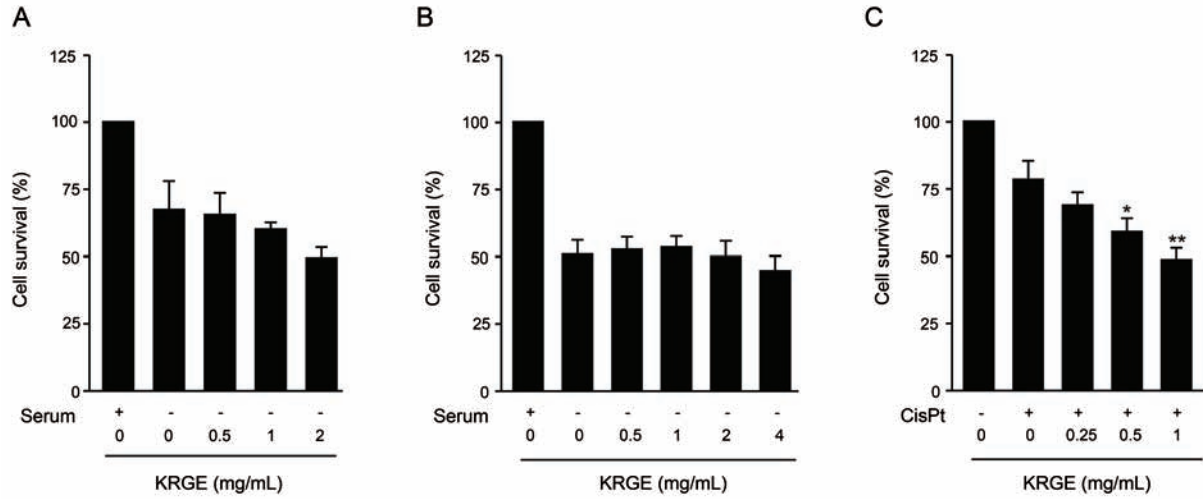
Fig. 1. Korean Red Ginseng extract (KRGE) protects human umbilical vein endothelial cells (HUVECs) from serum deprivation-induced cell death. (A) HUVECs were cultured in serum-free M199 with 0.5 mg KRGE/mL or 5% fetal bovine serum (FBS)-containing M199 as a control. After the indicated time periods, cell viability was determined by CCK-8 assay. (B) Under the same experimental condition, cell viability was also determined after incubation for 48 h by microscopy. (C) Cells were treated with the indicated concentrations of KRGE in serum-free M199 for 48 h. Cell viability was determined by CCK-8 assay. Data shown are the mean±SD ($n≥3$). *$p<0.05$ and **$p<0.01$ vs. serum-free condition without KRGE. (D) Cells were treated with the indicated with KRGE or American ginseng extract (AGE) in serum-free media for 48 h, and cell viability was determined. Data shown are the mean±SD ($n≥3$). *$p<0.05$.

KRGE elicits antiapoptotic activity by inhibiting the mitochondrial apoptotic pathway

To verify whether KRGE regulates apoptotic or necrotic cell death of endothelial cells cultured in serum-free conditions, we examined the effects of caspase inhibitors on KRGE-mediated protection against serum deprivation-induced endothelial cell death. Endothelial cell death induced by serum deprivation was effectively blocked by the pan-caspase inhibitor z-VAD-fmk and the caspase-3 inhibitor Ac-DEVD-cho, but not by the caspase-8 inhibitor Ac-IETD-cho (Fig. 3A). Moreover, KRGE showed a similar protective effect to that of Ac-DEVD-cho (Fig. 3A). These results suggest that KRGE protects HUVECs from serum deprivation-induced apoptotic cell death. Since apoptosis can occur either via the

intrinsic pathway that is triggered by death receptor-mediated caspase-8 activation and Bid cleavage or through the extrinsic pathway that is induced by mitochondrial cytochrome *c* relocalization [14], we next examined which pathway is responsible for KRGE-mediated antiapoptosis. Treatment with KRGE suppressed mitochondrial cytochrome *c* release into the cytosol, without increasing caspase-8-like protease (IETDase) activity and Bid cleavage (Fig. 3B, C). Cytosolic cytochrome *c* is known to play a crucial role in the activation of the initiator caspase-9 and the effector caspase-3. Thus, we further examined the effect of KRGE on caspase-9/-3 activities in serum-deprived HUVECs. As expected, KRGE significantly decreased serum deprivation-induced increases in casepase-9-like protease (LEHDase) activity, similar

Fig. 2. Korean Red Ginseng extract (KRGE) does not protect against tumor cell death. (A) B16F1 and (B) HeLa cells were incubated in serum-free media containing the indicated concentrations of KRGE or media containing 5% fetal bovine serum (FBS) for 48 h. (C) HeLa cells were cultured with 10 μg/mL of cisplatin alone or in combination with KRGE in Dulbecco's modified Eagle's medium containing 5% FBS for 24 h. Cell viability determined by CCK-8 assay. All data shown are the mean±SD (*n*=3). *$p<0.05$ and **$p<0.01$ vs. cisplatin alone.
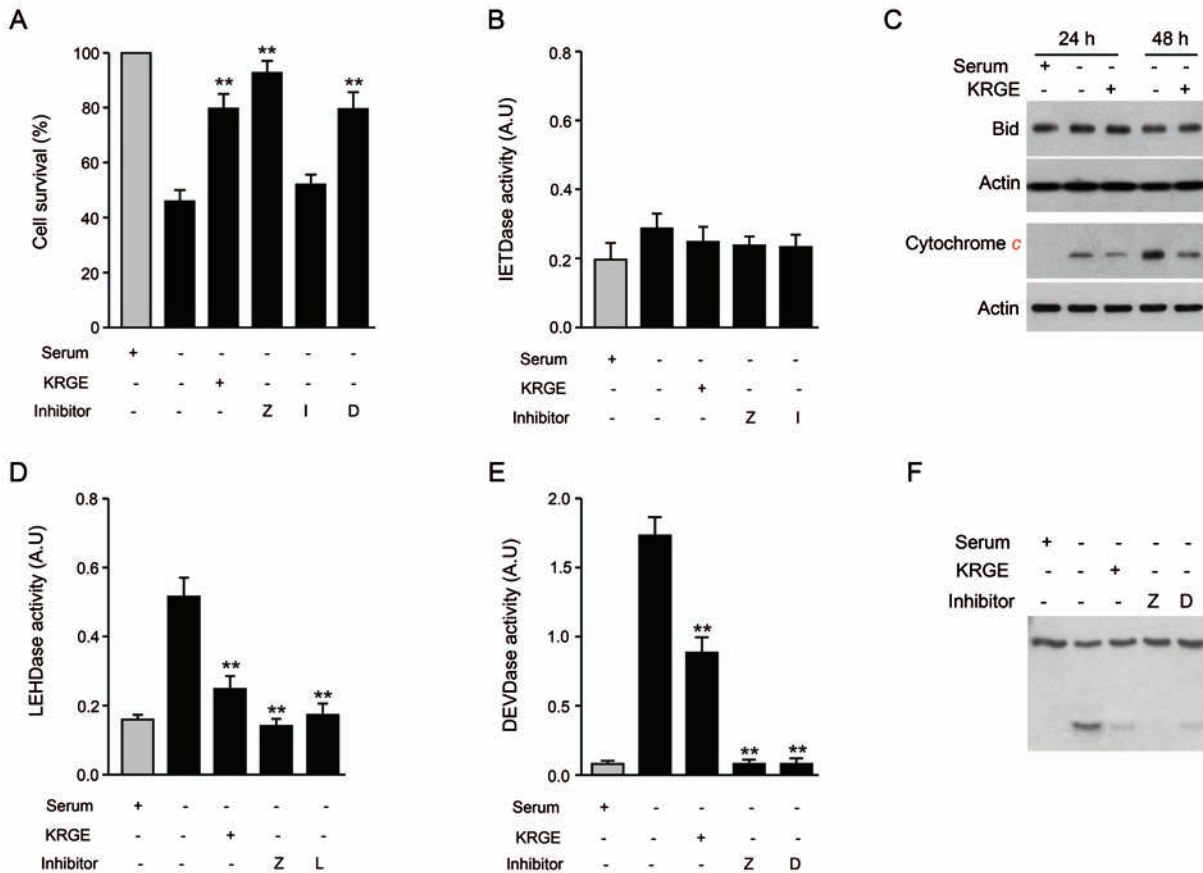
Fig. 3. Korean Red Ginseng extract (KRGE) inhibits caspase-dependent endothelial cell apoptosis, without caspase-8 activation. Human umbilical vein endothelial cells (HUVECs) were treated with 0.5 mg/mL KRGE, 100 μM Z-VAD-fmk (Z), 100 μM Ac-LEHD-cho (L), 100 μM Ac-IETD-cho (I), or 100 μM Ac-DEVD-cho (D) in serum-free media. (A) After 48 h, cell viability was measured by CCK-8 assy. (B) IETDase activity was determined in the cell lysates from HUVECs treated with KRGE in the presence or absence of caspase inhibitors for 36 h by colorimetric assay. (C) Protein levels of Bid and cytochrome *c* were determined in the cell lysates and cytosolic fractions by Western blotting. (D,E) LEHDase and DEVDase activities were determined in the cell lysates by colorimetric assay. (F) Caspase-3 activation was determined in cell lysates by Western blot analysis. All graphic data shown are the mean±SD (*n*=3). **$p<0.01$ vs. serum-deprived cells.

to the effect of Z-VAD-fmk and the caspase-9 inhibitor Ac-LEHD-cho (Fig. 3D). Moreover, KRGE markedly suppressed caspase-3-like protease (DEVDase) activity and proteolytic activation of caspase-3, and these effects were slightly lower than those of z-VAD-fmk and Ac-DEVD-cho (Fig. 3E, F). These results suggest that KRGE inhibits serum deprivation-induced activation of the mitochondria-dependent extrinsic apotosis pathway in human endothelial cells.

KRGE increases antiapoptotic Bcl-2 family protein expression and Bad phosphorylation

We next examined whether KRGE regulates the expression of Bcl-2 family proteins, which are important regulators of apoptosis [15]. KRGE treatment elevated the expression of the antiapoptotic Bcl-2 and Bcl-X_L proteins, but not the proapoptotic Bad and Bax proteins (Fig. 4A). Since the biological function of the proapoptotic molecule Bad is regulated by PI3K/Akt-dependent phosphorylation, we analyzed whether KRGE regulates Bad phosphorylation using an antibody specific to phosphorylated Bad at Ser-136. KRGE treatment led to increased Bad phosphorylation in a dose-dependent manner (Fig. 4B). We further confirmed a role of the PI3K/Akt pathway in KRGE-induced Bad phosphorylation using the PI3K inhibitor Wortmannin. KRGE significantly increased Akt and Bad phosphorylation, and these effects were suppressed by treatment with Wortmannin (Fig. 4C). Antiapoptotic and proapoptotic Bcl-2 family pro-

teins are mutually redistributed between the mitochondrion and the cytosol following an apoptotic stimulus and activate the mitochondria-dependent apoptotic pathway [15]. We therefore examined the effect of KRGE on the subcellular redistribution of Bcl-2 family proteins. KRGE prevented the cytosolic redistribution of Bcl-2 and Bcl-X_L from mitochondria after serum deprivation, thereby retaining their mitochondrial levels. However, KRGE inhibited mitochondrial translocation of Bax and Bad from the cytosol, and these effects were significantly reversed by Wortmannin (Fig. 4D). In addition, the suppressive effect of KRGE on mitochondrial cytochrome *c* release was also attenuated by Wortmannin (Fig. 4D). These data suggest that KRGE inhibits subcellular redistribution of Bcl-2 family proteins between the mitochondrion and the cytosol by up-regulating Bcl-2 and Bcl-X_L, in addition to its ability to increase Akt-dependent Bad phosphorylation.

KRGE increases Akt-dependent endothelial nitric oxide synthase (eNOS activation and NO production

Since the PI3K/Akt pathway is involved in the regulation of endothelial cell function, such as vasorelaxation and cell survival, via activation of the eNOS/NO pathway [16], we examined whether KRGE regulates Akt activation and eNOS activity in human endothelial cells. Treatment of HUVECs with KRGE significantly increased Akt phosphorylation in both a dose- and time-
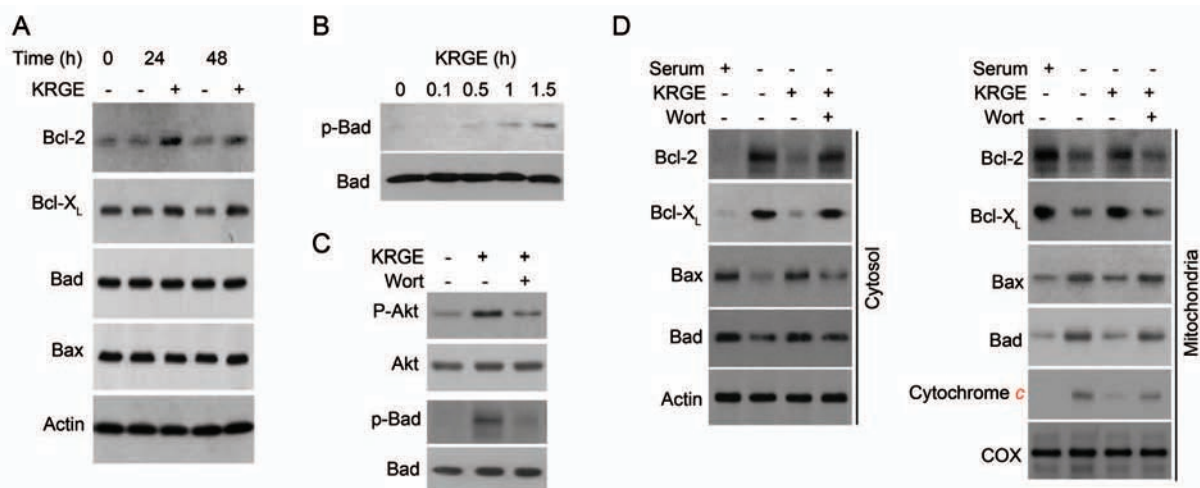
Fig. 4. Korean Red Ginseng extract (KRGE) up-regulates antiapoptotic proteins Bcl-2 and Bcl-X_L and phosphorylates Bad. (A) Human umbilical vein endothelial cells (HUVECs) were treated with or without 0.5 mg/mL KRGE for the indicated time periods. The levels of Bcl-2 family proteins were determined by Western blot analysis. (B) Phospho-Bad (p-Bad) level was determined in cell lysates from HUVECs treated with 0.5 mg/mL KRGE for the indicated time periods. (C) Phosphorylation of Akt and Bad was determined in cell lysates from HUVECs treated with 0.5 mg/mL KRGE in the presence or absence of Wortmannin (Wort, 50 nM) for 1.5 h. (D) Cytosolic and mitochondrial fractions were isolated from HUVECs treated with KRGE in the presence or absence of Wort. The levels of Bcl-2 family proteins were determined by Western blotting. Cytochrome *c* oxidase (COX) was used as an internal control of mitochondrial fractions.
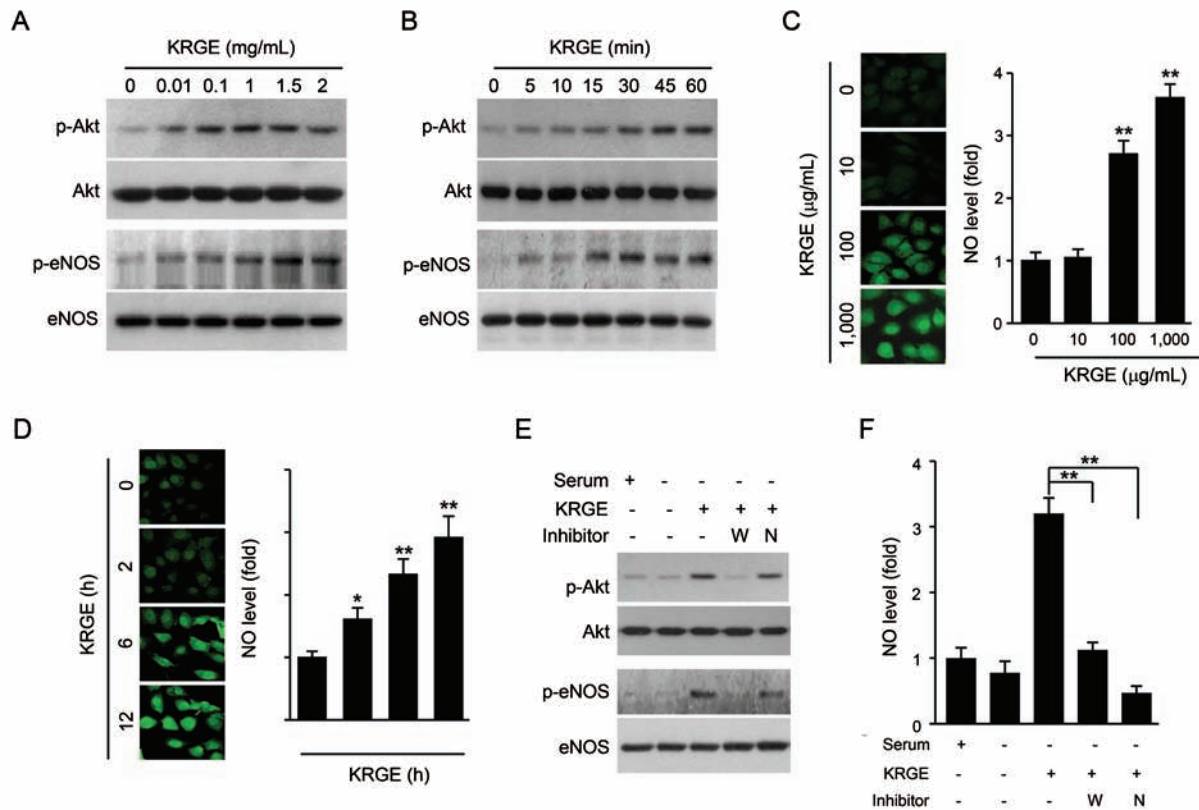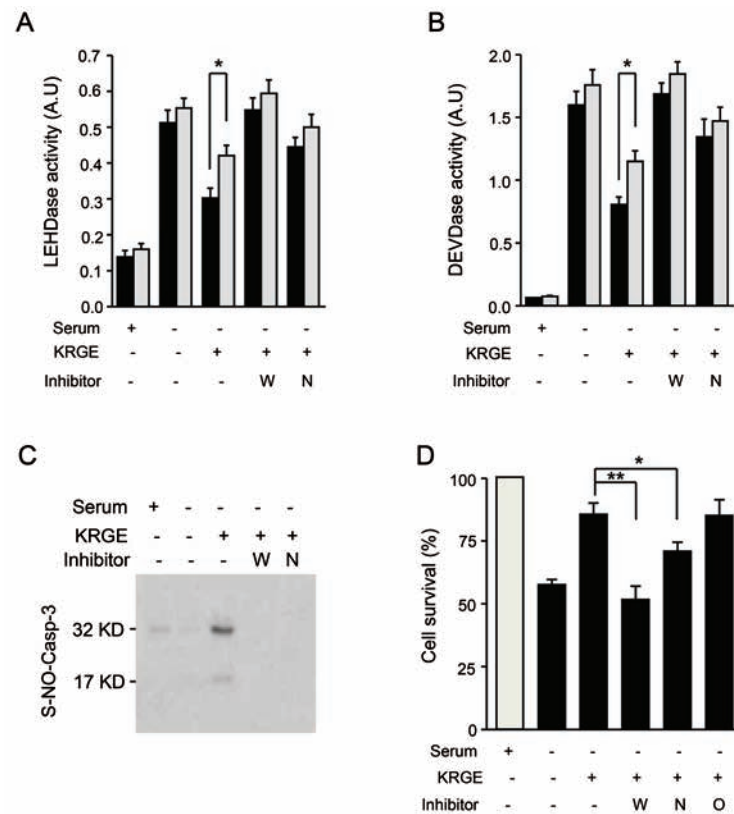
Fig. 5. Korean Red Ginseng extract (KRGE) increases Akt-dependent endothelial nitric oxide synthase (eNOS) activation and nitric oxide (NO) production. (A,B) Human umbilical vein endothelial cells (HUVECs) were treated with the indicated concentrations or 0.5 mg/mL KRGE for 1 h or the indicated time periods in M199 with 5% fetal bovine serum. Phosphorylation of Akt (p-Akt) and eNOS (p-eNOS) was determined by Western blot analysis. (C,D) Intracellular NO levels were determined in HUVECs treated with the indicated concentrations or 0.5 mg/mL of KRGE for 1 h or the indicated time periods by confocal microscopy. Data shown are the mean±SD (n=3). *p<0.05 and **p<0.01 vs. control. (E) Cells were treated with 0.5 mg/mL KRGE in the presence or absence of Wortmannin (W, 50 nM) and NAM (N, 1 mM) for 1 h. Phosphorylation of Akt and eNOS was determined by Western blotting. (F) Under the same experimental condition, intracellular NO levels were determined by confocal microscopy. Data shown are the mean±SD (n=3). *p<0.05 and **p<0.01.

dependent manner, which were highly correlated with eNOS phosphorylation (Fig. 5A, B). We next determined the intracellular accumulation of NO in HUVECs treated with KRGE by confocal microscopy and quantified relative NO levels. Treatment with KRGE increased intracellular NO levels in both a dose- and time-dependent manner (Fig. 5C, D). KRGE-induced Akt phosphorylation and eNOS activation were effectively blocked by the addition of Wortmannin, but not by the eNOS inhibitor NMA (Fig. 5E). In addition, KRGE-mediated NO production was suppressed by Wortmannin and NMA (Fig. 5F). These results suggest that KRGE increased NO production in HUVECs by increasing PI3K/Akt-dependent eNOS activation.

KRGE prevents HUVEC apoptosis via *S*-nitrosylation of caspases by elevating NO production

To examine a role of NO in regulating caspase activity via *S*-nitrosylation, we determined caspase activity in cell lysates, which were preincubated with or without 20 mM DTT, a reducing agent that removes the NO group from S-nitrosothiols. Caspase-9/-3 activities in cytosolic extracts from HUVECs stimulated with KRGE were nearly 50% lower than those in serum-deprived cells, and these inhibitory effects were reversed in HUVECs treated with Wortmannin or NMA (Fig. 6A, B). Preincubation of the cytosolic extracts with DTT significantly restored KRGE-induced inhibition of caspase-9/-3 activities (Fig. 6A, B). Furthermore, the biotin switch assay showed that KRGE increased *S*-nitrosylation of procaspase-3 and active caspase-3, and this modification was blocked by treatment with Wortmannin or NMA (Fig. 6C). As expected, the KRGE-mediated antiapoptotic effect was blocked by Wortmannin and NMA, but not by the addition of the soluble guanylyl cyclase inhibitor ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one) (Fig. 6D). These results suggest that KRGE also protects endothelial cells from serum deprivation-induced apoptosis

Fig. 6. Korean Red Ginseng extract (KRGE) prevents endothelial cell apoptosis via *S*-nitrosylation of caspases by increasing nitric oxide (NO) production. (A,B) Human umbilical vein endothelial cells (HUVECs) were treated with 0.5 mg/mL of KRGE in the presence or absence of Wortmannin (W, 50 nM) and NMA (N, 1 mM) for 36 h. The cell lysates were preincubated with or without 20 mM dithiothreitol, and LEHDase and DEVDase activities were determined by colormetric assay. Data shown are the mean±SD (n=3). *p<0.05. (C) *S*-nitrosylation of caspase-3 was determined in the cell lysates from HUVECs treated with KRGE in the presence or absence of Wortmannin and NMA for 18 h by biotin-switch assay. (D) Cells were treated with KRGE in the presence or absence of W, N, or ODQ (O, 20 µM) for 48 h. Cell death was determined using Cell Counting Kit 8. Data shown are the mean±SD (n=3). *p<0.05 and **p<0.01.
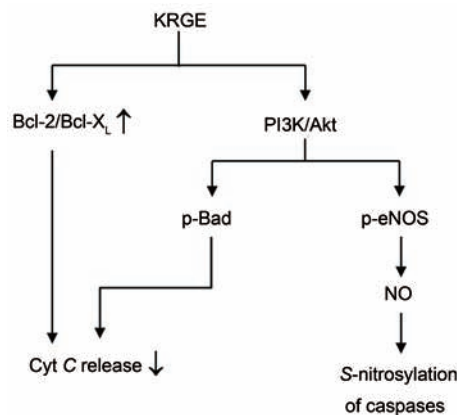
Fig. 7. The possible antiapoptotic mechanism induced by Korean Red Ginseng extract (KRGE). eNOS, endothelial nitric oxide synthase; NO, nitric oxide.

via *S*-nitrosylation of caspase-9/-3 by increasing eNOS-dependent NO production, but not by cyclic guanosine monophosphate (cGMP) synthesis.

DISCUSSION

The root of *P. ginseng* is known to show a wide spectrum of medicinal effects, such as anti-inflammatory, antitumorigenic, antidiabetic, and antiaging activities [17]. Ginsenosides as active components of ginseng products contribute to the improvement of vascular function. Indeed, the ginsenosides, including Rg1 and protopanaxatriol, are shown to protect endothelial cells from apoptosis induced by β-amyloid peptide or oxidative stress [18,19]. Our previous study also demonstrated that Rg3 prevents endothelial cells from serum-derived apoptosis via Akt-dependent inhibition of the mitochondrial apoptosis pathway [20]. However, the protective and antiapoptotic effects of KRGE, a mixture of ginsenosides, in serum-deprived HUVECs and its underlying mechanism have not been extensively studied. We here found that KRGE protected against caspase-mediated apoptosis of HUVECs, but not tumor cells in a serum-

deprived culture condition, which is similar to *in vitro* simulation of pathological ischemia. This differential effect may be due to KRGE-mediated increases in NO production in endothelial cells, but not in tumor cells, and NO-mediated *S*-nitrosylation of caspases, leading to inactivation of caspases [21]. Importantly, our data also show that KRGE has a greater protective effect on serum deprivation-induced apoptosis than AGE. This differential protective effect of these extracts may be due to their distinct ginsenoside profiles [22]. For example, the ginsenosides F4, Rg3, and Rg5 were newly synthesized in Korean Red Ginseng produced by steaming process [23], in addition to 13 ginsenosides that are uniquely found in Korean Red Ginseng [24]. Although the antiapoptotic function of these ginsenosides has not been clearly elucidated, they are thought to contribute to the protective effect of KRGE. These evidences indicate that KRGE elicits a protective role against serum deprivation-induced endothelial cell apoptosis, but not tumor cells, whose activity is significantly higher than that of AGE.

There are two distinct apoptosis pathways, namely the death receptor-mediated (extrinsic) and mitochondria (intrinsic) pathways [14]. Cross-linking of death receptors with their respective ligands (e.g., CD95L and TRAIL) induces sequential intracellular signal events, such as caspase-8 activation, Bid cleavage, mitochondrial cytochrome *c* release, and caspase-9/-3 activation, resulting in cleavage of target proteins to induce apoptosis. On the other hand, intrinsic death stimuli (e.g., cytotoxic chemicals) induce cytochrome *c*-mediated apoptic signal cascade, without caspase-8 activation. Both signal pathways are integrated in mitochondrial dynamics, including subcellular redistribution of mitochondrial cytochrome *c* and converge on the common downstream effector caspase-3. We found that serum-deprived HUVECs increased mitochondrial cytochrome *c* release and caspase-9/-3 activation, without caspase-8-mediated cleavage of Bid, suggesting that serum deprivation induces endothelial cell apoptosis via the intrinsic pathway [20]. Moreover, endothelial cell apotosis induced by serum deprivation was effectively inhibited by KRGE, but not by a caspase-8 inhibitor. These data indicate that KRGE protects HUVECs from intrinsic apoptosis induced by serum deprivation.

Mitochondrial cytochrome *c* release is a key step in the activation of the extrinsic and intrinsic pathways of apoptosis, and this event can be regulated by the expression levels of Bcl-2 family proteins and their post-translational modification [4,25]. The antiapoptotic Bcl-2 and Bcl-X_L proteins are predominantly localized in the

mitochondria and prevent mitochondrial dysfunction. On the other hand, the proapoptotic Bad and Bax proteins are normally localized in the cytosol. Following an intrinsic death signal, pro-apoptotic and antiapoptotic Bcl-2 family proteins redistribute between the mitochondrion and the cytosol. These signals result in the increase of mitochondrial proapoptotic Bcl-2 levels and decrease of mitochondrial antiapoptotic Bcl-2 members, which subsequently increase cytochrome *c* release from mitocondria. Thus, the cellular ratio of antiapoptotic and proapoptotic Bcl-2 members is an important determinant of mitochonndrial cytochrome *c* release. The present study showed that KRGE significantly increased the total levels of Bcl-2 and Bcl-X_L, but not Bad and Bax, resulting in an increase in cellular ratio of antiapoptotic to proapoptotic Bcl-2 family proteins. Indeed, KRGE blocked serum deprivation-induced subcellular redistribution of proapoptotic and antiapoptotic Bcl-2 family proteins between the mitochondria and the cytosol, leading to prevention of mitochondrial cytochrome *c* release. These results suggest that KRGE protects HUVECs from serum deprivation-induced apoptosis by up-regulating the antiapoptotic Bcl-2 family proteins (Fig. 7).

Posttranslational modification of proapoptotic Bcl-2 members, such as Bad phosphorylation and Bid cleavage, is well analyzed for their role in regulating mitochondria-dependent apoptosis. Cleavage of cytosolic Bid by caspase-8 generates a carboxy-terminal fragment, called truncated Bid, which translocates to the mitochondria and promotes cytochrome *c* release from the mitochondria [26]. However, we did not observe caspase-8 activation and Bid cleavage in serum-deprived endothelial cells, indicating that caspase-8-mediated Bid cleavage is not involved in serum deprivation-induced HUVEC apoptosis. On the other hand, the serine/threonine kinase Akt, downstream of PI3K, phosphorylates Bad on two serine residues (Ser-112 and Ser-136), which is sequestered in the cytosol by the phosphoserine-binding protein 14-3-3 [27]. KRGE promoted Akt activation and Bad phosphorylation, which were highly correlated with suppression of mitochondrial Bad translocation and cytochrome *c* release. Importantly, these cellular events were signficantly inhibited by a PI3K inhibitor, indicating the critical involvement of the PI3K/Akt pathway in KRGE-induced Bad phosphorylation and antiapoptosis (Fig. 7).

Endothelial NO production plays an important role in controlling various vascular functions, including apoptosis [28]. KRGE can increase endothelial NO synthesis by post-translational eNOS activation via Ca^{2+}-sensitive eNOS dimerization and Akt-dependent eNO

phosphorylation [11,29,30]. We have previously shown that NO protectes endothelial cells from apoptosis under different conditions [7,8,31,32]. These studies directed our attention toward NO as a potential mediator of KRGE-induced antiapoptosis. Importantly, the present study revealed that KRGE increased NO production from HUVECs by increasing Akt-dependent eNOS phosphorylation, without significant increasing eNOS protein. However, the possibilty that KRGE promotes eNOS dimerization cannot be excluded, because we did not directly determine intracellular Ca^{2+} levels and eNOS dimerization.

Although high levels of NO are shown to be cytotoxic [33], physiological levels of NO can prevent apoptosis in several cell types, including endothelial cells and hepatocytes [32,34]. Several mechanisms for the antiapoptotic effect of NO have been identified, including elevation of *S*-nitrosylation of caspases and cGMP-dependent protien kinase acrivation [34]. We here found that the antiapoptotic effect of KRGE was attenuated by Wortmannin and NMA, suggesting that the antiapoptotic effect of KRGE is linked to the Akt/eNOS/NO pathway. However, we did not observe any inhibition of the antiapoptotic effect of KRGE with an inhibitor against soluble guanylyl cyclase, indicating that cGMP does not act as a critical mediator of the KRGE/eNOS/NO-dependent protective pathway. NO-mediated antiapoptosis involves direct inhibition of caspase activation/activity via *S*-nitrosylation of the inactive and active forms of caspases [34,35]. We found that inhibition of caspase-9/-3 activities by KRGE was reversed by eNOS inhibition. Furthermore, the inhibitory effect of KRGE on these caspase activities was partially reversed by pre-incubating cell lysates with DTT, suggesting that KRGE-mediated increases in NO inhibit these caspases by redox-sensitive *S*-nitrosylation of their catalytic cysteine residues. These results are similar to our previous observation that *S*-nitrosylation of caspase-3 by NO inhibits TNFα-induced apoptosis [34]. Indeed, we directly confirmed that KRGE increased *S*-nitrosylation of pro- and active caspase-3, which was inhibited by Wortmannin and NMA. It is likely that the *S*-nitrosylated caspases cannot elicit their downstream signal cascade and prevent apoptotic cell death. Thus, KRGE promotes cell survival in serum-deprived HUVECs via *S*-nitrosylation of caspases by increasing eNOS-derived NO production (Fig. 7).

In conclusion, we demonstrated the cytoprotective effect of KRGE on serum deprivation-induced HUVEC apoptosis and its underlying mechanism linked to multiple signal pathways to include up-regulation of antiapoptotic Bcl-2 family proteins, Akt-dependent Bad and eNOS phosphorylation, elevation of endothelial NO production, and redox-sensitive *S*-nitrosylation of caspase-9/-3 (Fig. 7). These findings indicate that KRGE protects HUVECs from apoptosis through regulation of Bcl-2 family dynamics and caspase *S*-nitrosylation mainly via PI3K/Akt-dependent and -independent pathways. The antiapoptotic property of KRGE may be valuable for developing new pharmaceutical that will protect against endothelial cell death at the site of vascular injury induced by atherosclerosis and stroke.

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REFERENCES

1. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 1998;91:3527-3561.
2. Stoneman VE, Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. Clin Sci (Lond) 2004;107:343-354.
3. Williams TA, Verhovez A, Milan A, Veglio F, Mulatero P. Protective effect of spironolactone on endothelial cell apoptosis. Endocrinology 2006;147:2496-2505.
4. Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. Trends Cell Biol 2000;10:369-377.
5. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes *in vitro* and protects against ischemia-reperfusion injury in mouse heart. Circulation 2000;101:660-667.
6. Stefanec T. Endothelial apoptosis: the missing link between atherosclerosis and SLE? Blood 2004;103:3608-3609.
7. Kim YM, Bombeck CA, Billiar TR. Nitric oxide as a bifunctional regulator of apoptosis. Circ Res 1999;84:253-256.
8. Lee SJ, Kim KM, Namkoong S, Kim CK, Kang YC, Lee H, Ha KS, Han JA, Chung HT, Kwon YG et al. Ni-

tric oxide inhibition of homocysteine-induced human endothelial cell apoptosis by down-regulation of p53-dependent Noxa expression through the formation of S-nitrosohomocysteine. J Biol Chem 2005;280:5781-5788.

9. Hoffmann J, Haendeler J, Aicher A, Rossig L, Vasa M, Zeiher AM, Dimmeler S. Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide. Circ Res 2001;89:709-715.

10. Sung J, Han KH, Zo JH, Park HJ, Kim CH, Oh BH. Effects of red ginseng upon vascular endothelial function in patients with essential hypertension. Am J Chin Med 2000;28:205-216.

11. Kim YM, Namkoong S, Yun YG, Hong HD, Lee YC, Ha KS, Lee H, Kwon HJ, Kwon YG, Kim YM. Water extract of Korean red ginseng stimulates angiogenesis by activating the PI3K/Akt-dependent ERK1/2 and eNOS pathways in human umbilical vein endothelial cells. Biol Pharm Bull 2007;30:1674-1679.

12. Lee SJ, Namkoong S, Kim YM, Kim CK, Lee H, Ha KS, Chung HT, Kwon YG, Kim YM. Fractalkine stimulates angiogenesis by activating the Raf-1/MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways. Am J Physiol Heart Circ Physiol 2006;291:H2836-H2846.

13. Qian J, Chen F, Kovalenkov Y, Pandey D, Moseley MA, Foster MW, Black SM, Venema RC, Stepp DW, Fulton DJ. Nitric oxide reduces NADPH oxidase 5 (Nox5) activity by reversible *S*-nitrosylation. Free Radic Biol Med 2012;52:1806-1819.

14. Crow MT, Mani K, Nam YJ, Kitsis RN. The mitochondrial death pathway and cardiac myocyte apoptosis. Circ Res 2004;95:957-970.

15. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999;13:1899-1911.

16. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399:601-605.

17. Lu JM, Yao Q, Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr Vasc Pharmacol 2009;7:293-302.

18. Yan J, Liu Q, Dou Y, Hsieh Y, Liu Y, Tao R, Zhu D, Lou Y. Activating glucocorticoid receptor-ERK signaling pathway contributes to ginsenoside Rg1 protection against β-amyloid peptide-induced human endothelial cells apoptosis. J Ethnopharmacol 2013;147:456-466.

19. Kwok HH, Ng WY, Yang MS, Mak NK, Wong RN, Yue PY. The ginsenoside protopanaxatriol protects endothelial cells from hydrogen peroxide-induced cell injury and cell death by modulating intracellular redox status. Free Radic

Biol Med 2010;48:437-445.

20. Min JK, Kim JH, Cho YL, Maeng YS, Lee SJ, Pyun BJ, Kim YM, Park JH, Kwon YG. 20(S)-ginsenoside Rg3 prevents endothelial cell apoptosis via inhibition of a mitochondrial caspase pathway. Biochem Biophys Res Commun 2006;349:987-994.

21. Dimmeler S, Haendeler J, Nehls M, Zeiher AM. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. J Exp Med 1997;185:601-607.

22. Sengupta S, Toh SA, Sellers LA, Skepper JN, Koolwijk P, Leung HW, Yeung HW, Wong RN, Sasisekharan R, Fan TP. Modulating angiogenesis: the yin and the yang in ginseng. Circulation 2004;110:1219-1225.

23. Kim WY, Kim JM, Han SB, Lee SK, Kim ND, Park MK, Kim CK, Park JH. Steaming of ginseng at high temperature enhances biological activity. J Nat Prod 2000;63: 1702-1704.

24. Kim SN, Ha YW, Shin H, Son SH, Wu SJ, Kim YS. Simultaneous quantification of 14 ginsenosides in *Panax ginseng* C.A. Meyer (Korean red ginseng) by HPLC-ELSD and its application to quality control. J Pharm Biomed Anal 2007;45:164-170.

25. Zinkel S, Gross A, Yang E. BCL2 family in DNA damage and cell cycle control. Cell Death Differ 2006;13:1351-1359.

26. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998;94:491-501.

27. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 1996;87:619-628.

28. Moncada S, Radomski MW, Palmer RM. Endothelium-derived relaxing factor. Identification as nitric oxide and role in the control of vascular tone and platelet function. Biochem Pharmacol 1988;37:2495-2501.

29. Shin W, Yoon J, Oh GT, Ryoo S. Korean red ginseng inhibits arginase and contributes to endotheliumdependent vasorelaxation through endothelial nitric oxide synthase coupling. J Ginseng Res 2013;37:64-73.

30. Hellermann GR, Solomonson LP. Calmodulin promotes dimerization of the oxygenase domain of human endothelial nitric-oxide synthase. J Biol Chem 1997;272:12030-12034.

31. Kwon YG, Min JK, Kim KM, Lee DJ, Billiar TR, Kim YM. Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production. J Biol Chem 2001;276:10627-10633.

32. Ceneviva GD, Tzeng E, Hoyt DG, Yee E, Gallagher A, Engelhardt JF, Kim YM, Billiar TR, Watkins SA, Pitt BR. Nitric oxide inhibits lipopolysaccharide-induced apoptosis in pulmonary artery endothelial cells. Am J Physiol 1998;275:L717-L728.

33. Granger DL, Taintor RR, Cook JL, Hibbs JB Jr. Injury of neoplastic cells by murine macrophages leads to inhibition of mitochondrial respiration. J Clin Invest 1980;65:357-370.

34. Kim YM, Talanian RV, Billiar TR. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. J Biol Chem 1997;272: 31138-31148.

35. Mannick JB, Hausladen A, Liu L, Hess DT, Zeng M, Miao QX, Kane LS, Gow AJ, Stamler JS. Fas-induced caspase denitrosylation. Science 1999;284:651-654.s