J Ginseng Res 45 (2021) 344–353

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research article

Korean Red ginseng prevents endothelial senescence by downregulating the HO-1/NF-κB/miRNA-155-5p/eNOS pathway

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ARTICLE INFO

Article history:
Received 15 January 2020
Received in Revised form
5 July 2020
Accepted 10 August 2020
Available online 14 August 2020

Keywords:
Endothelial cells
Endothelial nitric oxide synthase
miR-155-5p
Panax ginseng
Senescence

ABSTRACT

Background: Korean Red ginseng extract (KRGE) has beneficial effects on the cardiovascular system by improving endothelial cell function. However, its pharmacological effect on endothelial cell senescence has not been clearly elucidated. Therefore, we examined the effect and molecular mechanism of KRGE on the senescence of human umbilical vein endothelial cells (HUVECs).
Methods: HUVECs were grown in normal or KRGE-supplemented medium. Furthermore, they were transfected with heme oxygenase-1 (HO-1) gene or treated with its inhibitor, a NF-κB inhibitor, and a miR-155-5p mimic or inhibitor. Senescence-associated characteristics of endothelial cells were determined by biochemical and immunohistochemical analyses.
Results: Treatment of HUVECs with KRGE resulted in delayed onset and progression of senescence-associated characteristics, such as increased lysosomal acidic β-galactosidase and decreased telomerase activity, angiogenic dysfunction, and abnormal cell morphology. KRGE preserved the levels of anti-senescent factors, such as eNOS-derived NO, MnSOD, and cyclins D and A: however, it decreased the levels of senescence-promoting factors, such as ROS, activated NF-κB, endothelial cell inflammation, and p21 expression. The beneficial effects of KRGE were due to the induction of HO-1 and the inhibition of NF-κB-dependent biogenesis of miR-155-5p that led to the downregulation of eNOS. Moreover, treatment with inhibitors of HO-1, NF-κB, and miR-155-5p abolished the anti-senescence effects of KRGE.
Conclusion: KRGE delayed or prevented HUVEC senescence through a signaling cascade involving the induction of HO-1, the inhibition of NF-κB-dependent miR-155-5p biogenesis, and the maintenance of the eNOS/NO axis activity, suggesting that it may protect against vascular diseases associated with endothelial senescence.

1. Introduction

Endothelial cells (ECs) that constitute the inner cellular lining of blood vessels have a key role in regulating vascular homeostasis and function, such as angiogenesis, vascular integrity, vasorelaxation, and local inflammation [1]. Their functioning is compromised in various pathophysiological conditions, including aging and cellular senescence. Thus, EC senescence is considered an important determinant of vascular dysfunction, which plays a crucial role in the development and progression of cardiovascular disorders.

Endothelial nitric oxide synthase (eNOS)-derived NO plays a crucial role in endothelial function and senescence associated with cardiovascular diseases, such as hypertension, atherosclerosis, and cardiac ischemia [2–4]. Although eNOS has been known to be constitutively expressed and activated by post-translational modifications [2], recent studies have demonstrated that its expression can be also regulated post-transcriptionally by nuclear factor-κB

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https://doi.org/10.1016/j.jgr.2020.08.002
p1226-8453 e2093-4947/$ – see front matter

(NF-κB), thus defining eNOS as a negative target gene of NF-κB [5,6]. The antioxidant protein heme oxygenase-1 (HO-1) has been shown to improve endothelial function by restoring the NF-κB-mediated eNOS downregulation through the production of carbon monoxide (CO) [7]. This study has suggested that the HO-1/CO axis prevents endothelial dysfunction and senescence by preserving and maintaining with the eNOS/NO pathway [8,9].

MicroRNAs (miRNAs or miRs) post-transcriptionally inhibit gene expression by binding to the 3′-untranslated region of specific mRNAs [10]. miRNAs play important roles in cardiovascular diseases and cellular senescence [11]. Several miRNAs, including NF-κB-responsive miR-31-5p and miR-155-5p, have been found to induce EC dysfunction via downregulation of eNOS, contributing to vascular diseases [5,6]. Thus, drugs or compounds that inhibit NF-κB activation may prevent endothelial senescence and dysfunction by rescuing miR-155-5p-mediated eNOS downregulation, thus subsequently improving vascular function and homeostasis.

Panax ginseng Meyer is a well-known oriental herbal medicine. Among many ginseng products, Korean Red ginseng (KRG), a ginseng root that is steamed and dried, has multiple pharmacological effects in a wide range of pathological conditions, such as cardiovascular and inflammatory diseases [4]. Accumulating evidence has suggested that KRG and its active ingredients, including Rb1, Rg5, and Rg3, exert beneficial actions on oxidative stress, apoptotic cell death, and EC function [12–15]. Ginseng products have been shown to regulate NF-κB activation, miRNA biogenesis, and the eNOS/NO pathway [16–18], probably by stimulating the HO-1/CO pathway [7]. However, the molecular mechanism and effect of KRG extract (KRGE) on EC senescence have not been clearly elucidated. Therefore, the aim of the present study is to investigate the molecular mechanism and pharmacological effect on replicative EC senescence.

2. Materials and methods

2.1. Materials

KRGE was supplied by the Korea Ginseng Cooperation (Daejeon, Korea). Briefly, KRG was produced by steaming 6-year-old fresh ginseng roots (P. ginseng Meyer) at 90–100°C for 3 h and drying them at 50–80°C. KRGE was prepared from KRG by circulating hot water (85–90°C) three times for 8 h. The water content of the pooled extract was 36% of the total weight. HPLC analysis showed that KRGE contained the following major ginsenosides: Rb1, 7.44 mg/g; Rb2, 2.59 mg/g; Rc, 3.04 mg/g; Rd, 0.91 mg/g; Re, 1.86 mg/g; Rf, 1.24 mg/g; Rg1, 1.79 mg/g; Rg2, 1.24 mg/g; and Rg3, 1.39 mg/g. KRGE was dissolved in medium 119 (M199) at a concentration of 500 μg/mL and filtered through a 0.22-μm sterile filter (Corning Inc., Corning, NY, USA). List and information of chemicals, antibodies, RNA, and other reagents used in this study were provided in the supplementary Table 1.

2.2. EC culture and cell proliferation assay

Human umbilical vein endothelial cells (HUVECs) were grown in M199 as previously described [6]. HUVECS were plated at a density of 1.5×10^4 cells/cm^2 in culture plates and grown in complete M199 supplemented with or without 0.5 mg/mL KRGE. Some cells were transfected with 80 nM of a miR-155-5p inhibitor or a miR-155-5p mimic using Lipofectamine RNAiMAX or treated with Tin protoporphyrin IX dichloride (SnPP, 10 μM), N^G-Monomethyl-L-arginine (NMA, 1 mM), or Bay11-7802 (5 μM) for the indicated time periods. Cell number was counted using a hematocytometer every 3 days or at the end of every passage when cells reached approximately 80% confluence.

2.3. SA-β-galactosidase (SA-β-gal) staining and telomerase activity assay

SA-β-gal staining was performed using a SA-β-gal Staining Kit according to the manufacturer's instructions. Telomerase activity was determined using the *Telo*TAGGG telomerase PCR ELISA^{PLUS} Kit according to the manufacturer's instructions. The protein concentration was determined by the Bradford assay.

2.4. Immunofluorescence staining

Cells were grown on glass cover slips and fixed with 4% formaldehyde for 10 min. The cells were permeabilized with 0.1% Triton X-100 for 5 min and then blocked with 1% bovine serum albumin/10% normal goat serum/0.3 M glycine in 0.1% phosphate-buffered saline (PBS)-Tween for 1 h. The cells were incubated overnight at 4°C with anti-β-tubulin antibody (1:100) conjugated with Fluor® 488. Nuclear DNA was counterstained with propidium iodide (PI) or 4′,6-diamidino-2-phenylindole (DAPI). Nuclear translocation of NF-κB p65 was assessed by immunostaining of cells with anti—NF—κB p65 antibody (1:100) in combination with rhodamine-conjugated anti-rabbit IgG (1:200).

2.5. Luciferase reporter assay

HUVECs were transfected with 0.5 μg pNFB-Luc plasmid (Stratagene, La Jolla, CA, USA) along with 0.5 μg of pCMV-LacZ by using a microporator (NanoEnTek, Seoul, Korea). Cells were cultured in complete M199 supplemented with or without 0.5 mg/mL KRGE for 48 h. Cell lysates were prepared with passive lysis buffer, and luciferase and β-gal activities were measured with a luciferase assay system and *ortho*-nitrophenyl-β-D-galactopyranoside, respectively. Luciferase reporter activity was normalized relative to β-gal activity.

2.6. Flow cytometry analysis

HUVECs (~1×10^6) were harvested with 1 mL of trypsin-EDTA and centrifuged at $1{,}000 \times g$ for 5 min at room temperature. Cells were incubated with an isotype control FITC-conjugated goat anti-rat IgG or a FITC-conjugated VCAM-1 antibody (1:100) for 30 min in ice-cold PBS containing 2% fetal bovine serum, fixed with 2% paraformaldehyde, and analyzed using a FACSCalibur flow cytometer (DB Bioscience, San Jose, CA, USA).

2.7. Monocyte-EC adhesion assay

HUVECs were plated in 2% gelatin-coated 96-well plates at a density of 1×10^4 cells/well and grown overnight to reach confluence. Human monocytic U937 cells were then added (5×10^4 cells/mL, 200 μL/well) to the confluent HUVEC monolayers and incubated for 1 h. Thereafter, the cells were washed three times with PBS, and the remaining cells were fixed and stained with Diff-Quick. Adherent cells were counted in five randomly selected optical fields of each well.

2.8. Angiogenesis assay

HUVECs grown in M199 supplemented with or without KRGE were transfected with control or miR-155-5p mimic for 3 days. HUVECs were stimulated with or without 10 ng/mL of vascular endothelial growth factor-A (VEGF-A), followed by measurement of their angiogenic properties, such as proliferation, migration, and tube formation, as described previously [5]. EC proliferation was determined by the [^{3}H]-thymidine incorporation assay. Cell

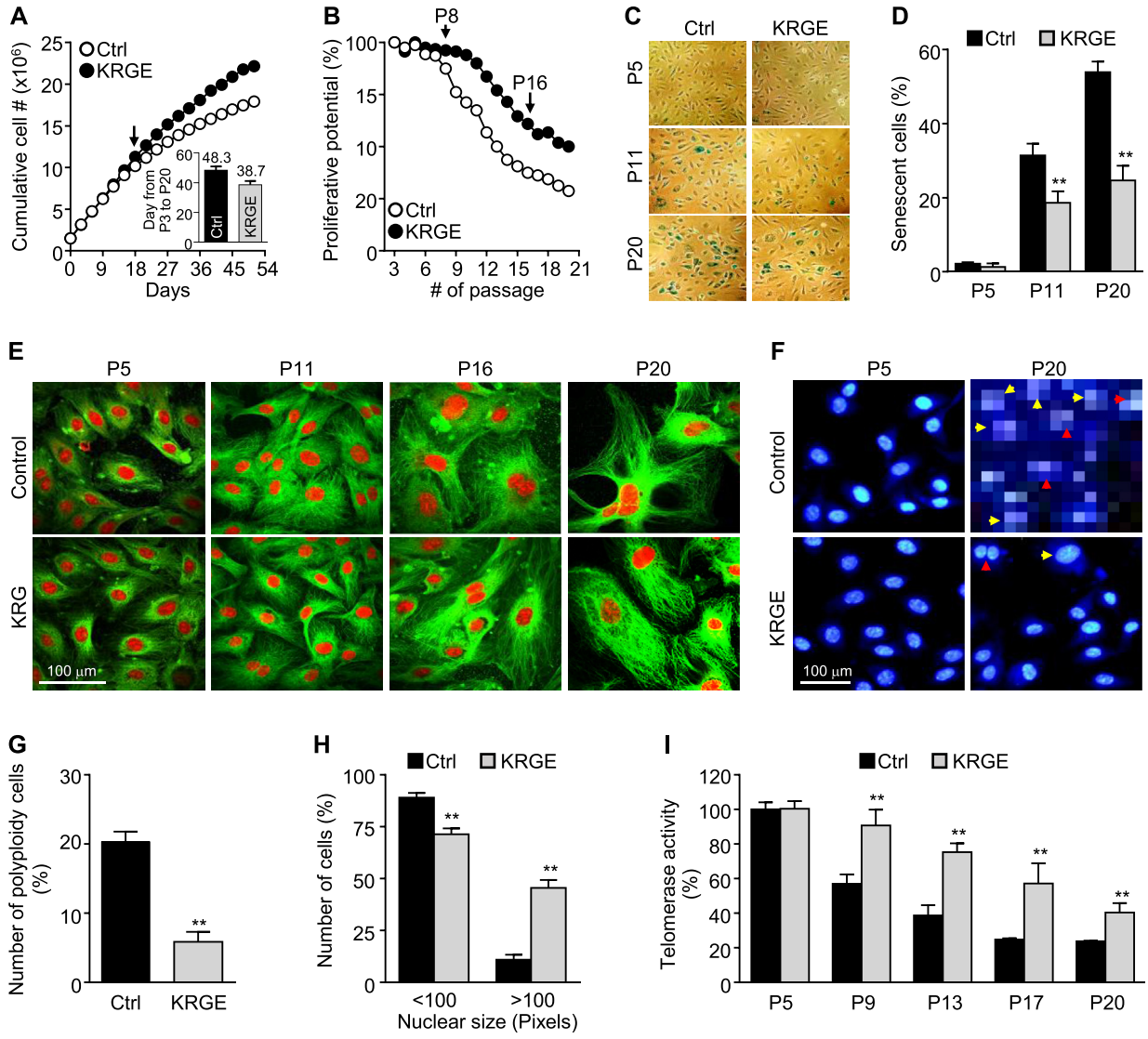

Fig. 1. KRGE suppresses replicative senescence of HUVECs. (A) Cell numbers were counted every 3 days using a hemocytometer (n = 2). Inserted graph: day to reach passage 20 from passage 3 (n = 3). (B) Cell numbers were counted at the end of every passage, and the proliferative potential was calculated based on cell counts (n = 2). (C and D) Cells were incubated with SA-β-gal staining solution at the indicated passages. Senescent cells were counted (n = 3). (E) HUVECs were stained with an anti-β-tubulin antibody (green) and PI (red). (F) Nucleus was stained with DAPI and photographed. Yellow and red arrows indicate enlarged and polyploid nuclei, respectively. (G and H) The number of cells with polyploidy and enlarged nuclei was counted at passage 20 (n = 3). (I) Telomerase activity was assayed using Telomerase PCR ELISA kit (n = 3). $^{**}P < 0.01$.

migration assay was performed using Boyden chambers (Corning Life Sciences, Tewksbury, MA, USA). Tube-like structure formation was determined after culturing HUVECs on a layer of growth factor-reduced Matrigel.

2.9. Measurements of intracellular reactive oxygen species (ROS) and NO

Intracellular ROS were measured as previously described [19]. Briefly, cells were incubated with 10 μM 2′,7′-dichlorodihydro-fluorescein diacetate (H_2DCFDA) for 1 h in serum-free medium. The cells were washed with PBS, and fluorescence intensity was visualized in randomly selected areas by confocal microscopy. In addition, intracellular NO levels were determined by confocal microscopy using 4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate) as previously reported [8]. Some cells at passage 20 were transfected with pDsRed-N1-HO-1 (HO-1 was cloned into the EcoR1/BamH1 sites of the pDsRed-N1 vector) and cultured in M199 for 48 h. The intracellular ROS and NO levels were calculated based on the increased fluorescence intensity of DCF (a reaction product of ROS and H_2DCF) and DAF/NO adduct, respectively.

2.10. Western blotting and electromobility shift assay (EMSA)

Whole cell lysates (40 μg protein) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were hybridized with antibodies against target proteins, and the protein bands were visualized by exposure to X-ray film following incubation with

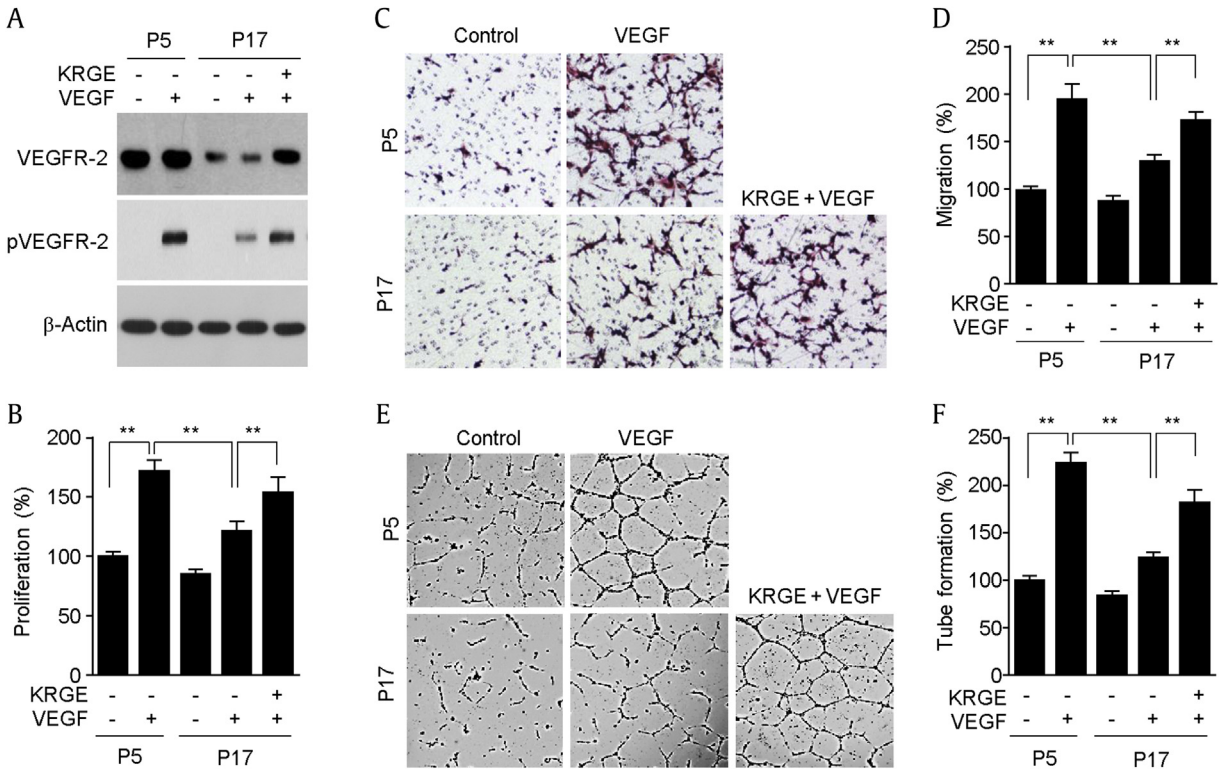

Fig. 2. KRGE recovers senescence-associated impairment of angiogenesis. (A) VEGFR-2 and phosphor-VEGFR-2 levels were determined by immunoblotting. (B) EC proliferation was determined by [^{3}H]-thymidine incorporation assay (n = 4). (C and D) Cell migration was assayed using Boyden chambers and quantified using ImageJ software (n = 4). (E and F) EC tube formation was determined on a layer of growth factor-reduced Matrigel and quantified using ImageJ software (n = 4). $^{**}P < 0.01$.

chemiluminescent reagents as previously described [20]. EMSA was performed as previously described [21]. In brief, a ^{32}P-labeled double stranded oligonucleotide (5′-AGTTGAGGGGACTTTCC-CAGGC-3′, ~40,000 cpm) was incubated with nuclear extracts (10 μg protein) in the presence or absence of an excess amount (100-fold) of cold oligonucleotide or an antibody for NF-κB p65 at room temperature for 20 min. Samples were separated on a 5% native polyacrylamide gel and subjected to autoradiography.

2.11. Measurement of miR-155-5p levels

Total miRNAs were isolated from HUVECs using a miRNeasy mini kit. cDNA for determining miRNAs was obtained from 1 μg of total RNA using a miScript II RT kit as previously described [5]. Quantitative real-time polymerase chain reaction analysis (qRT-PCR) was performed with miScript SYBR Green PCR Kit according to the manufacturer's instructions. The levels of miR-155-5p were analyzed by the miScript Primer Assay using a target miR-155-5p-specific primer and a universal primer. The relative levels of miRNA-155-5p were normalized to the housekeeping gene SNORD-95.

2.12. Statistical analysis

Quantitative data are expressed as the mean ± SEM of at least three separate experiments performed in triplicate. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad, Inc., La Jolla, CA, USA). Statistical significance was determined using either one-way ANOVA with Tukey's multiple comparisons test or the student's *t*-test, depending on the experimental groups analyzed. Significance was established at a P value < 0.05.

3. Results

3.1. KRGE delays cellular senescence of HUVECs

To assess the effect of KRGE on replicative senescence of ECs, we examined the effect of KRGE on the proliferative capacity of HUVECs. The cumulative number of HUVECs grown in the normal medium was proportionally increased by 18 days and thereafter their proliferation was gradually decreased. In contrast, the cumulative number of cells treated with 0.5 mg/mL of KRGE, which was determined based on the results of our previous study on endothelial cell function [22], was similar to that of non-treated control cells by 18 days and thereafter significantly higher than that of the control cells (Fig. 1A). As a result, serially-passaged HUVECs grown in the absence of KRGE needed 48.3 days to reach passage 20 from passage 3, while cells treated with KRGE needed 38.7 days (Fig. 1A. inserted graph). Similarly, HUVECs grown in normal medium showed a sudden decrease in proliferative potential after passage 8, whereas the cells grown in KRGE-supplemented medium maintained their proliferative potential until passage 11, and thereafter their proliferative capability slowly decreased (Fig. 1B). We further analyzed the senescence marker SA-β-gal activity in HUVECs grown in both media. SA-β-gal activity was significantly increased in HUVECs grown in the absence of KRGE in a passage-dependent manner, and the enzyme activity was attenuated in cells grown in the presence of KRGE (Fig. 1C and D). We also determined whether KRGE delayed senescence-associated morphological and biochemical changes. HUVECs grown in the absence of KRGE became flattened and enlarged, which are typical morphological characteristics of senescent cells [23]; however, the morphological alterations were delayed by growing cells in the presence of KRGE (Fig. 1E). HUVECs grown in the absence of KRGE

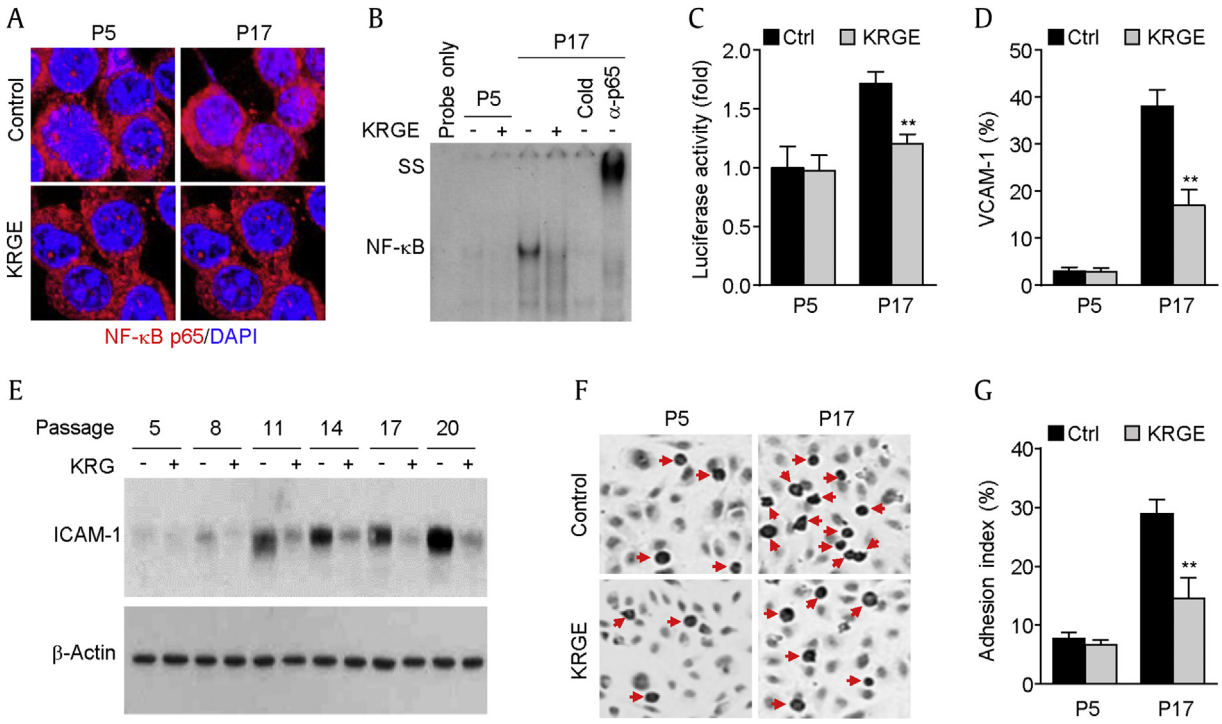

Fig. 3. KRGE attenuates senescence-associated NF-κB activation and endothelial inflammation. (A) HUVECs were stained with an antibody for NF-κB p65 and DAPI. (B) Nuclear NF-κB activity was analyzed by EMSA in the presence or absence of an excess amount of cold probe (cold) or an antibody for the NF-κB p65 subunit (α-p65). SS indicates super shift of the complex of NF-κB/DNA probe. (C) NF-κB-Luc activity was assayed in cell lysates by a luminometer (n = 3). (D) VECAM-1 expression levels were analyzed by flow cytometry (n = 3). (E) ICAM-1 levels were determined in HUVEC extracts by immunoblotting. (F and G) Monocyte-EC interaction was imaged by microscopy. Monocytic attachment (arrow) was quantified (n = 3). $^{**}P < 0.01$.

up to passage 20 also showed increased numbers of cells with polyploidy and enlarged nuclei, which are also known as characteristics of senescent cells [24,25], and this increase was inhibited by supplementation with KRGE (Fig. 1F–H). We further examined whether KRGE regulates telomerase activity that counteracts telomere shortening during cellular senescence [3]. Telomerase activity was decreased in serially passaged HUVECs in normal medium, which was prevented by KRGE supplementation (Fig. 1I). These results suggest that KRGE prevents or delays the replicative senescence of ECs. We also defined HUVECs at a passage lower than 6 and at a passage higher than 16 as young and senescent cells, respectively.

3.2. KRGE rescues senescence-associated impairment of angiogenesis

Since senescent ECs impair angiogenesis by altering the intracellular angiogenic signal cascade and/or downregulating the expression of angiogenesis- and senescence-associated receptors [26], we examined the expression of the representative key angiogenic receptor VEGF receptor-2 (VEGFR-2) and its activity in senescent ECs. HUVECs grown in the absence of KRGE at passage 17 markedly decreased VEGFR-2 levels compared to young cells at passage 5, and this decrease was rescued by KRGE supplementation, resulting in an increase in VEGF-A-induced phosphorylation of VEGFR-2 (Fig. 2A). As a result, senescent HUVECs at passage 17 decreased angiogenic properties, such as proliferation, migration, and tube formation, in response to VEGF-A compared to young cells, which was recovered by KRGE supplementation (Fig. 2B–F). These results suggest that KRGE prevents senescence-induced endothelial dysfunction by preventing VEGFR-2 downregulation.

3.3. KRGE inhibits NF-κB activation and inflammation

The NF-κB signaling pathway is activated and then induces inflammation during progression of cellular senescence [27]. We examined whether KRGE inhibits senescence-associated NF-κB activation and inflammation. Senescent HUVECs at passage 17 displayed NF-κB activation, as demonstrated by the nuclear translocation of the NF-κB subunit p65, the specific NF-κB-DNA binding activity in nuclear extracts via a cold probe-based competitive assay, and an antibody-based supershift assay; however, these events were inhibited by KRGE supplementation (Fig. 3A and B). As expected, KRGE supplementation blocked senescence-mediated increase in NF-κB-responsive promoter activity (Fig. 3C). We next examined the effect of KRGE on the expression of NF-κB-target genes, including adhesion molecules. KRGE supplementation significantly prevented upregulation of VCAM-1 and ICAM-1 in senescent HUVECs, as confirmed by flow cytometry and immunoblotting (Fig. 3D and E). As a result, KRGE suppressed the adhesive interaction of senescent HUVECs and monocytic U937 cells (Fig. 3F and G). Collectively, these results suggest that KRGE inhibits or delays NF-κB activation and inflammation in senescent HUVECs.

3.4. KRGE upregulates antioxidant and cell-cycle regulatory genes

Since oxidative stress causes replicative and cellular senescence [28], we examined the effect of KRGE on antioxidant gene expression in senescent ECs. The expression levels of manganese superoxide dismutase (MnSOD), but not Cu/ZnSOD and catalase, were significantly decreased in HUVECs at passages 11 and 17, however, treatment with KRGE restored its levels (Fig. 4A). Expression of HO-1, another antioxidant gene, was strongly induced by KRGE in HUVECs between passages 5 and 17 (Fig. 4A). As a result, HUVECs at

passage 17 markedly increased intracellular ROS levels, which were markedly reduced by KRGE supplementation (Fig. 4B and C). We further examined the effect of KRGE on the expression of cell cycle regulatory genes, including p53, p21, and cyclins, which are crucially involved in cellular senescence [23]. KRGE supplementation blocked upregulation of the cyclin-dependent kinase inhibitor p21 and elevated the expression levels of cyclins D and A in senescent HUVECs, whereas the expression levels of p53 were unaltered (Fig. 4D). These results suggest that KRGE inhibits oxidative stress and promotes cell cycle progression in ECs, leading to prevention of EC senescence.

3.5. KRGE rescues the eNOS/NO pathway

Since eNOS-derived NO functions as an anti-senescent factor in ECs [3], we examined the effect of KRGE on the eNOS/NO pathway. The expression levels of eNOS were significantly decreased in HUVECs at passages 11 and 17, and this decrease was rescued by KRGE (Fig. 5A and B). Consequently, a decrease in NO production in senescent HUVECs was nearly restored to the level observed in young cells by KRGE supplementation (Fig. 5C). Since the HO-1/CO pathway inhibits NF-κB-mediated downregulation of eNOS in HUVECs exposed to TNF-α [8], we examined whether KRGE-induced HO-1 regulates eNOS expression and NO production in senescent HUVECs. Treatment with the HO-1 inhibitor SnPP reversed KRGE-mediated prevention of eNOS downregulation in senescent HUVECs (Fig. 5D). Treatment of senescent HUVECs with the HO-1 inducer hemin elevated HO-1 expression and rescued the decreased levels of eNOS protein and NO production in senescent HUVECs (Fig. 5E and F). Moreover, mock-transfected senescent HUVECs at passage 17 markedly decreased intracellular NO production compared to young ECs at passage 5, and the decreased NO levels were largely reversed by transfecting with HO-1 gene. However, these effects were inhibited by co-treatment with SnPP or the NOS inhibitor NAM (Fig. 5G). These results suggest that KRGE has anti-senescent activity by upregulating HO-1, which rescues the decreased eNOS/NO axis activity in serially passaged HUVECs.

3.6. Anti-senescent effect of KRGE is associated with a HO-1/NF-κB/miR-155-5p/eNOS axis

Since miR-155-5p is an NF-κB-responsive miRNA that regulates eNOS expression [5,6,8], we examined the effect of KRGE on miR-155-5p synthesis in senescent ECs. miR-155-5p levels were increased in senescent HUVECs compared to young cells, and this increase was blocked by KRGE or the NF-κB inhibitor Bay11-708211 (Fig. 6A). The suppressive effect of KRGE on miR-155-5p biogenesis was reversed by SnPP (Fig. 6B). Notably, the recovery effect of KRGE on eNOS downregulation in senescent HUVECs was blocked by a miR-155-5p mimic, and senescence-induced eNOS downregulation was recovered by a miR-155-5p inhibitor (Fig. 6C). Similarly, KRGE-induced recovery of NO production in senescent HUVECs was blocked by a miR-155-5p mimic or SnPP (Fig. 6D). Moreover, the preventive effect of KRGE on increased SA-β-gal activity in senescent HUVECs was blocked by a miR-155-5p mimic or SnPP (Fig. 6E and F). Collectively, these results suggest that the anti-senescent effect of KRGE in HUVECs is associated with sequential events, such as induction of HO-1 and inhibition of NF-κB-dependent miR-155-5p, which targets the eNOS/NO pathway.

4. Discussion

Among many ginseng species, Korean ginseng might have a pharmacological effectiveness superior to that of others. Approximately 40 ginsenosides have been identified as the main bioactive ingredients in white ginseng [29]. Additionally, the ginsenosides Rh2, Rh4, Rs3, Rs4, Rg3, and Rg5 are newly synthesized during the steaming and drying process of ginseng and found only in red ginseng [30]. Although all the compounds exhibit their own biological activities, they can generate synergistic, additive, or increased effects when combined or mixed. Indeed, total ginseng extracts are more potent and beneficial than a single ginsenoside or the combination of certain ginsenosides [31]. Here, we examined the anti-senescent effect of KRGE and its underlying mechanism in HUVECs grown in the presence or the absence of KRGE.

Endothelium is composed of an inner monolayer of ECs in the vasculature and regulates vascular function and blood pressure. Thus, endothelial dysfunction is a crucial risk factor for cardiovascular diseases, such as hypertension, atherosclerosis, stoke, and diabetic retinopathy. Although endothelial dysfunction occurs due to various cytotoxic agents, including ROS and drugs, it can also naturally occur due to aging-associated endothelial senescence [32]. Thus, we examined the effect of KRGE on proliferative senescence of HUVECs, as an *in vitro* model of aging-related endothelial dysfunction. Our data showed that treatment of HUVECs with KRGE delayed development of senescence-associated characteristics, leading to improvement in angiogenesis and reduction in vascular inflammation. This evidence suggests that KRGE has a protective effect against endothelial senescence, although its pharmacological effect has not been examined in animal models or humans. In addition, based on evidence that red ginseng has more bioactive ginsenosides (e.g., Rg3 and Rg5) with antioxidant activity and anti-senescent effect of dermal fibroblasts than white ginseng [30,33,34], our results suggest that KRG has more preventive activity against endothelial senescence and vascular aging than white ginseng.

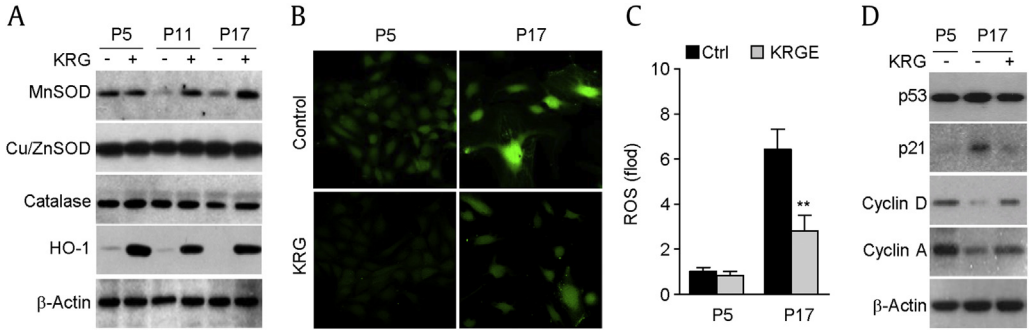

Fig. 4. KRGE regulates the expression of antioxidant and cell cycle-related genes. (A) Antioxidant protein levels were determined in HUVEC extracts by immunoblotting. (B and C) Intracellular ROS levels were determined by confocal microscopy using the ROS probe H_2DCFDA and quantified from the changes in fluorescence intensity (n = 10). (E) Cell cycle-related proteins levels were determined by immunoblotting. $^{**}P < 0.01$.

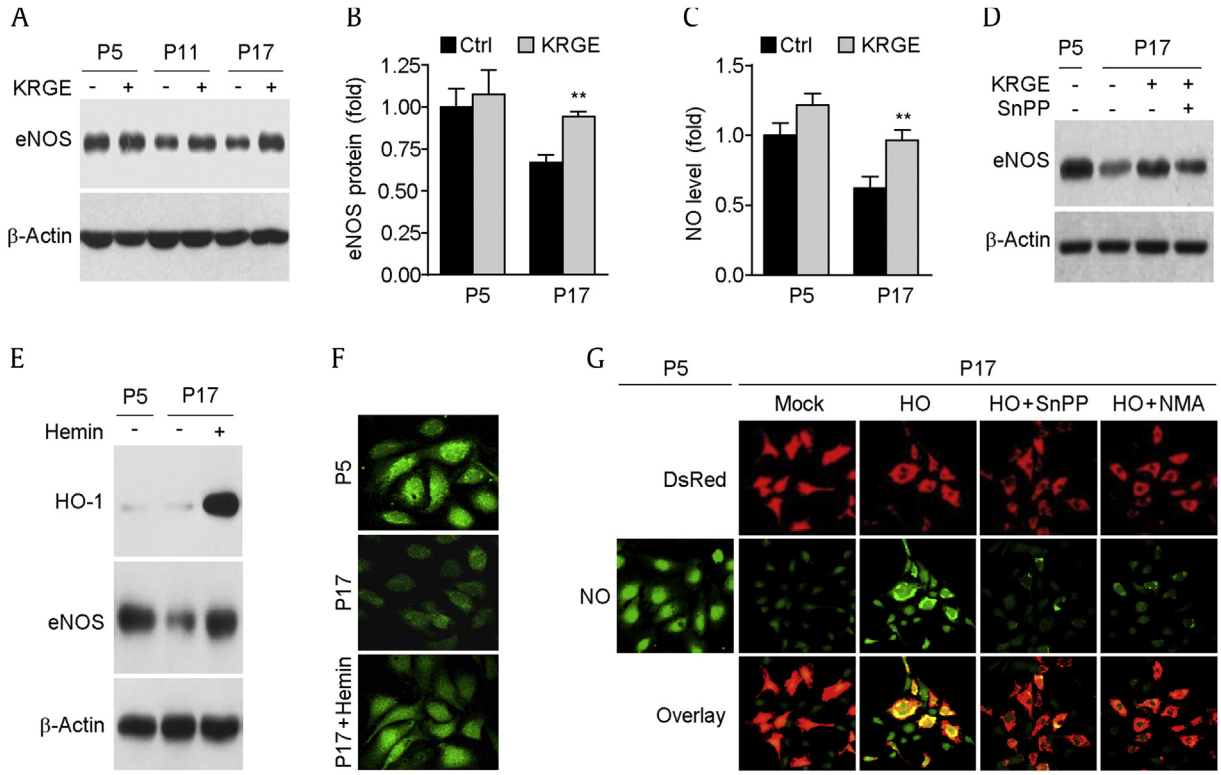

Fig. 5. KRGE rescues downregulated eNOS in senescent HUVECs in a HO-1-dependent manner. (A and B) eNOS levels were determined and quantified by immunoblotting (n = 3). (C) NO levels were measured and quantified using DAF-FM (n = 7). (D) Cells were treated with SnPP for 72 h. eNOS levels were determined by immunoblotting. (E and F) HUVECs were treated with 0.5 μM of hemin for 36 h. HO-1 and eNOS levels were determined by immunoblotting. NO levels were determined by confocal microscopy. (G) HUVECs were transfected with or without a pDsRed–HO–1 (HO) vector. Fluorescence images for NO and DsRed were observed by confocal microscopy. $^{**}P < 0.01$.

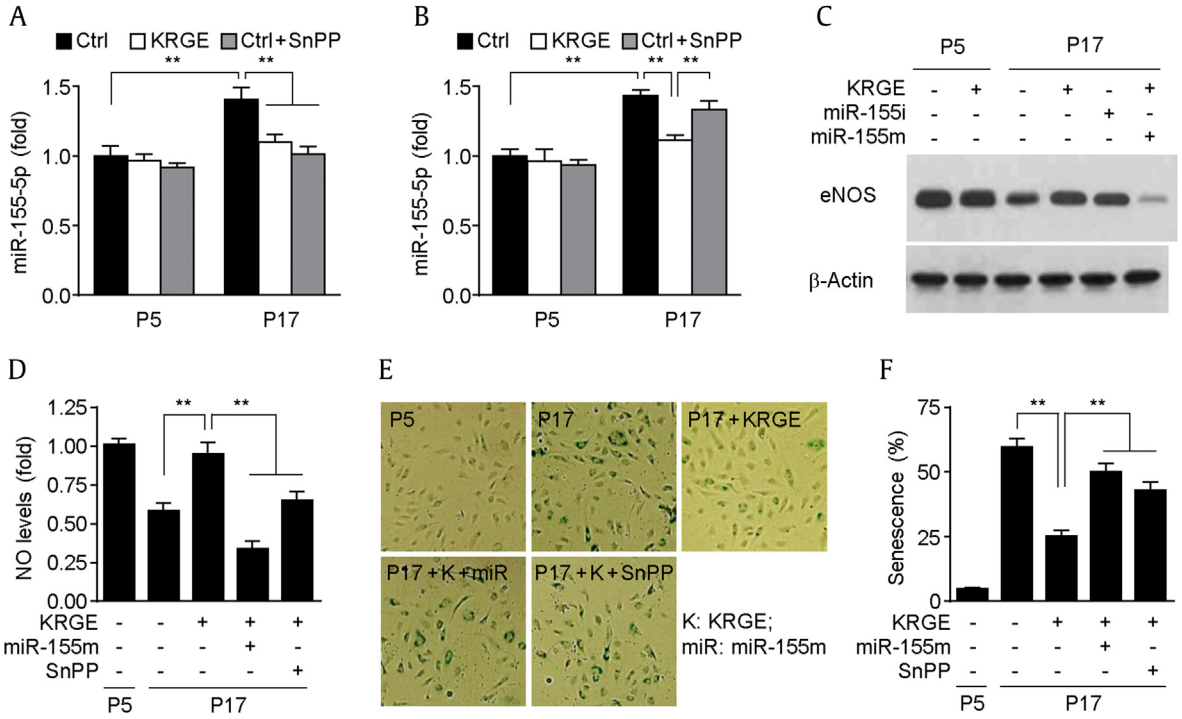

Fig. 6. KRGE inhibits endothelial senescence by regulating the HO-1/NF-κB/miR-155-5p/eNOS axis. HUVECs were grown in medium supplemented with or without KRGE until the indicated passage, followed by treatment with Bay11-7082 for 48 h, SnPP for 72 h, miR-155-5p mimic (miR-155m), or miR-155-5p inhibitor (miR-155i) for 72. (A and B) miR-155-5p levels were determined by qRT-PCR (n = 3). (C and D) eNOS levels were determined by immunoblotting, and intracellular NO levels were quantified by confocal microscopy (n = 4). (E and F) Senescent cell number was determined by counting SA-β-gal-positive cells (n = 4). $^{**}P < 0.01$.

Cellular senescence can be divided into two different types: replicative and stress-induced premature senescence. Replicative senescence is a fundamental feature of somatic cells, which have a limitation in cell division or replication, probably due to ROS accumulation and NF-κB activation, whereas premature senescence occurs in young proliferative cells exposed to adverse environmental conditions, such as oxidative stress [35]. Indeed, antioxidants, including *N*-acetylcysteine, delay the onset of replicative EC senescence by counteracting increased ROS production [36]. KRGE and ginsenosides have been shown to exert an antioxidant activity by directly scavenging ROS or upregulating the expression of antioxidant genes [37]. Ginsenosides, including Rb1, have been also shown to protect EC function by inhibiting H_2O_2-induced premature senescence [12,13]. Therefore, dietary intake of KRGE can suppress UV irradiation-induced senescence of dermal fibroblasts and delayed onset of skin wrinkles in a mouse model [38]. Unfortunately, its anti-senescent effect has not been determined in ECs. Our results demonstrate that KRGE prevents cellular senescence in serially passaged HUVECs through the reduction in intracellular ROS levels by inducing HO-1 expression and maintaining MnSOD levels.

Intracellular ROS accumulation is widely observed in cellular senescence and activates NF-κB, suggesting that ROS is sufficient to activate NF-κB, which acts as a master regulator of senescence-associated secretory phenotype, including production of inflammatory cytokines [36]. In addition, aging- or senescence-mediated activation of NF-κB is associated with increased ROS production [39], and endothelial senescence is quickly induced by exposure to oxidative stress [12]. Accumulating evidence has demonstrated that NF-κB activation and inflammatory gene expression increased with aging and cellular senescence and elevated in aged human and rodents [40–42]. This suggests that the ROS/NF-κB axis plays an important role in cellular senescence. Thus, antioxidants or NF-κB inhibitors can attenuate cellular senescence in primary mouse embryonic fibroblasts exposed to oxidative stress [36,41]. Because KRGE has multiple biological functions, including direct antioxidant activity and upregulation of antioxidant genes [37], it can prevent endothelial senescence by suppressing oxidative stress. Our data demonstrate that KRGE delays senescence-associated characteristics of HUVECs by decreasing ROS generation and NF-κB activation via anti-oxidant gene expression. However, we cannot exclude the possibility that intrinsic antioxidant properties of KRGE are involved in its anti-senescent effect on endothelial cells. Therefore, the preventive effect of KRGE on endothelial senescence may be a result of the combination of intrinsic antioxidant activity and antioxidant gene expression.

In addition to direct scavenging of ROS [37], KRGE and other ginseng products have indirect antioxidant effects through activation of the transcription factor Nrf, resulting in the upregulation of antioxidant genes, including HO-1 and MnSOD [7,37,43]. Notably, HO-1-mediated CO production alleviates EC senescence by enhancing eNOS phosphorylation and NO production [9], suggesting that the HO-1/CO axis is coupled with the eNOS/NO pathway. Our previous study has shown that the HO-1/CO axis inhibited NF-κB activation and eNOS downregulation in HUVECs stimulated with TNF-α [8]. The present data demonstrate that KRGE alleviates senescence of ECs by upregulating HO-1, which subsequently prevents NF-κB activation and eNOS downregulation.

Although NF-κB activation has been shown to stimulate cellular senescence in fibroblasts and epithelial cells [27,36], the underlying mechanism has not been not clearly elucidated in ECs. In contrast to ROS, eNOS-derived NO functions as an inhibitor of endothelial senescence via activation of telomerase activity and suppression of SA-β-gal activity, leading to stimulation of cell proliferation [3,44]. Therefore, exogenous NO inhibited endothelial senescence, resulting in the prevention of vascular dysfunction associated with atherosclerosis and diabetes [3]. Notably, eNOS expression was downregulated in senescent ECs [44]. Although eNOS is constitutively expressed in ECs, recent studies have demonstrated that this gene is post-transcriptionally downregulated in an NF-κB-dependent manner [5,6]. This suggests that ROS-dependent NF-κB activation is crucially involved in senescence of ECs by suppressing eNOS-derived NO production. Similarly, we found that NF-κB activation and eNOS downregulation occurred in senescent HUVECs, which were rescued by KRGE, suggesting that the anti-senescent effect of KRGE is associated with the rescue of NF-κB-dependent eNOS downregulation.

The decreased eNOS expression has been directly linked to NF-κB-dependent biogenesis of miR-155-5p [5,8]. A pharmacological inhibitor of NF-κB and silencing of the NF-κB subunit p65 prevented downregulation of eNOS expression by suppressing miR-155 synthesis. Moreover, HO-1 overexpression and exogenous CO have been shown to attenuate TNF-α-induced endothelial dysfunction by blocking NF-κB-dependent miR-155-5p biogenesis and impairing the eNOS/NO pathway [8]. This suggests that the HO-1/CO axis crucially contributes to the prevention of EC senescence by maintaining the eNOS/NO axis activity and downregulating NF-κB-dependent miR-155-5p biogenesis. In agreement with this suggestion, our data showed that NF-κB-responsive miR-155-5p was upregulated in senescent HUVECs and inhibited the eNOS/NO pathway, which were blocked via HO-1 induction in HUVECs grown in the presence of KRGE. These findings suggest that KRGE inhibits NF-κB-dependent miR-155-5p biogenesis and maintains the eNOS/NO axis activity, resulting in a delay or prevention of HUVEC senescence.

EC senescence is a key step in endothelial dysfunction, which is a hallmark of cardiovascular diseases, including hypertension and atherosclerosis. Since endothelium-derived NO plays a crucial role in vasorelaxation, anti-inflammation, and angiogenesis, the impaired eNOS/NO pathway caused by senescence or aging is a pathogenic risk factor for cardiovascular disorders [26]. Notably, miR-155-5p has been shown to promote hypertension, atherosclerosis, and endothelial dysfunction via post-transcriptional inhibition of eNOS expression [5,8,45]. In addition, the VEGR-2/VEGF-A axis also plays an important role in EC function related to angiogenesis. We found that VEGFR-2 was downregulated in senescent HUVECs, resulting in the impairment of VEGF-A-induced angiogenesis, which was recovered following their treatment with KRGE. This is in agreement with previous reports that VEGFR-2 expression is suppressed in aged mice and can be regulated in an NO-dependent manner [46–48]. This suggests that impairment of the eNOS/NO pathway in senescent HUVECs results in VEGFR-2 downregulation, subsequently decreasing angiogenesis-associated wound healing in patients with diabetes, which accelerates EC senescence [49]. These data suggest that KRGE prevents senescence-mediated VEGFR-2 downregulation in HUVECs by suppressing NF-κB-dependent biogenesis of miR-155-5p, which downregulates eNOS expression.

Taken together, the present data indicate that KRGE treatment results in a delay of EC senescence. The anti-senescent action of KRGE is mediated by sequentially inducing HO-1, removing ROS, suppressing NF-κB-dependent miR-155-5p biogenesis, and impairing the eNOS/NO pathway. Although further animal studies on the pharmacological benefits are needed, the anti-senescent effect of KRGE on ECs suggests that it may have therapeutic effect on endothelial dysfunction-associated diseases, including hypertension and atherosclerosis.

Conflict of interest

All authors have no conflicts of interest to declare.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grants funded by the Ministry of Science, ICT and Future Planning (MSIP; NRF-2017R1A2B3004565).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2020.08.002.

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