

Korean Red Ginseng Water Extract Restores Impaired Endothelial Function by Inhibiting Arginase Activity in Aged Mice

Kwanhoon Choi^{2*}, Jeongyeon Yoon^{1*}, Hyun Kyo Lim², and Sungwoo Ryoo¹

¹Department of Biology, Kangwon National University, Chuncheon 200-701, ²Department of Anesthesiology and Pain Medicine, Yonsei University Wonju College of Medicine, Wonju 220-701, Korea

Cardiovascular disease is the prime cause of morbidity and mortality and the population ages that may contribute to increase in the occurrence of cardiovascular disease. Arginase upregulation is associated with impaired endothelial function in aged vascular system and thus may contribute to cardiovascular disease. According to recent research, Korean Red Ginseng water extract (KRGE) may reduce cardiovascular disease risk by improving vascular system health. The purpose of this study was to examine mechanisms contributing to age-related vascular endothelial dysfunction and to determine whether KRGE improves these functions in aged mice. Young (10±3 weeks) and aged (55±5 weeks) male mice (C57BL/6J) were orally administered 0, 10, or 20 mg/mouse/day of KRGE for 4 weeks. Animals were sacrificed and the aortas were removed. Endothelial arginase activity, nitric oxide (NO) generation and reactive oxygen species (ROS) production, endothelial nitric oxide synthase (eNOS) coupling, vascular tension, and plasma peroxynitrite production were measured. KRGE attenuated arginase activity, restored nitric oxide (NO) generation, reduced ROS production, and enhanced eNOS coupling in aged mice. KRGE also improved vascular tension in aged vessels, as indicated by increased acetylcholine-induced vasorelaxation and improved phenylephrine-stimulated vasoconstriction. Furthermore, KRGE prevented plasma peroxynitrite formation in aged mice, indicating reduced lipid peroxidation. These results suggest KRGE exerts vasoprotective effects by inhibiting arginase activity and augmenting NO signaling and may be a useful treatment for age-dependent vascular diseases.

Key Words: Aging, Endothelial nitric oxide synthase, Korean red ginseng extract, Vasorelaxation

INTRODUCTION

Cardiovascular disease is the primary cause of morbidity and mortality in both industrialized and developing countries [1]. The occurrence of cardiovascular disease is going to be increased as the people ages, although successful treatments for cardiovascular risk factors, such as hypertension, hypercholesterolemia, and atherosclerosis, are rapidly developing.

The hallmark of the aging-related cardiovascular system is a decrease in nitric oxide (NO) bioavailability [2,3] and an


increase in reactive oxygen species (ROS) production [4,5]. Superoxide ($O_2^{\cdot-}$) is a free radical that decreases NO bioavailability by rapidly scavenges NO. NO and $O_2^{\cdot-}$ also can react to generate peroxynitrite ($ONOO^-$), a highly oxidant molecule to macromolecules such as protein and DNA [6]. Simultaneous generation of NO and $O_2^{\cdot-}$ can raise $ONOO^-$ to levels potentially harmful to homeostasis of vascular cell [6], and the imbalance of nitroso-redox system contributes to age-related endothelial dysfunction and vascular stiffness [7].

Nitric oxide (NO) is a major factor in endothelium-dependent vasorelaxation of the vascular system. NO act as a potent vasoprotective molecule by regulating vasoreactivity, inhibiting platelet activation and leukocyte adhesion, and preventing smooth muscle cell proliferation and migration in vasculature. NO is generated from its substrate L-arginine by eNOS enzyme in the endothelium and plays critical roles in the maintenance of vascular homeostasis. Arginase shares a common substrate L-arginine with eNOS and hydrolyzes L-arginine to L-ornithine and urea at the

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Corresponding to: Sungwoo Ryoo, Department of Biology, Kangwon National University, Kangwondae-gil 1, Chuncheon 200-701, Korea. (Tel) 82-33-250-8534, (Fax) 82-33-251-3990, (E-mail) ryosw08@kangwon.ac.kr, Hyun Kyo Lim, Department of Anesthesiology and Pain Medicine, Yonsei University Wonju College of Medicine, 162 Ilsan-dong, Wonju 220-701, Korea. (Tel) 82-33-741-1536, (Fax) 82-33-742-8198, (E-mail) hyunkylim@yonsei.ac.kr

*The first two authors contributed equally to this work.

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ABBREVIATIONS: KRGE, Korean Red Ginseng Extract; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; DAF-FM, 4-Amino-5-methylamino-2', 7'-difluorofluorescein diacetate; DHE, dihydroethidine; PE, phenylephrine; ODQ, 1H-[1,2,4]Oxadiazolo [4,3-a] quinoxalin-1-one; Ach, acetylcholine; TBA, Thiobarbituric acid; SNP, sodium nitroprusside.

last step of the urea cycle. It is well documented that arginase reciprocally regulates NOS activity by modulating intracellular L-arginine bioavailability [7]. Thus, the balance of activities between these enzymes, arginase and eNOS, reciprocally regulates endothelial NO production.

Korean Red Ginseng water extract (KRGE) has been extensively studied, and its consumption has progressively increased in the general population. Ginseng has beneficial effects for treating various diseases including thrombosis, hyperlipidemia, cancer, and atherosclerosis [8-12]. KRGE exerts vasoprotective effects by eliminating $O_2^{\cdot -}$ derived from NADPH oxidase [13], promoting endothelial cell proliferation and protecting against H_2O_2 -dependent cell death [9,14], and inducing heme oxygenase-1 expression [15]. Ginseng extract exerts a direct vasodilatory effect by releasing NO in an endothelium-dependent manner [16]. Ginseng may exert its effects by activating phosphoinositide 3-kinase (PI3K)/Akt signal transduction in endothelial cells, which stimulates eNOS to produce NO, thereby leading to vascular system improvements [17].

Age-dependent vascular changes have been investigated in humans and a number of other species [18]. However, the degree to which these dysregulated mechanisms contribute to age-related vascular pathology remain to be elucidated. The contribution of vascular control mechanisms in healthy, aging, and disease conditions affected by vessel type and size [18]. In a previous study, we demonstrated that KRGE regulates NO production and enhances NO signaling in wild-type young mice [19]. The goal of this study was to investigate which mechanisms contribute to age-related endothelial dysfunction in aged mice and determine whether orally administered KRGE improves age-associated impairments in endothelial function.

METHODS

Materials

KRGE (Fig. 1, solid extract 64% from 6-year old ginseng roots, Rg1, 1.7 mg/g, Rb1 8.4 mg/g, number of product, 2020013) was obtained from Korea Ginseng Corporation (Daejeon, Korea) and directly dissolved in distilled water. Mn(III) Tetra (4-benzoic acid) porphyrin chloride (MnTBAP) and N^G -nitro-L-arginine methyl ester (L-NAME) were purchased from Calbiochem. All reagents were from Sigma unless otherwise stated.

Animal protocol

Twenty young (10 ± 3 weeks, 17.7 ± 1.5 g) and twenty aged (55 ± 5 weeks, 29.3 ± 1.2 g) male mice (C57BL/6J) were used for all experiments. Mice were housed at 23°C under a 12-h light/12-h dark cycle. All animals had access to water and food (Nara Biotech.) *ad libitum*. KRGE was orally administered at a dose of 0, 10, or 20 mg/day for 4 weeks. Given that each mouse consumed approximately 10 ml water/day, this represented a daily dose of ~ 20 mg/mouse/day of KRGE. The study protocols were approved in accordance with the Guide for the Care and Use of Laboratory Animals (Institutional Review Board, Kangwon National University).

Arginase activity assay

Aorta tissue lysates were prepared in lysis buffer (50 mM

Tris-HCl, pH 7.5, 0.1 mM EDTA, and protease inhibitors). Lysates were homogenized at 4°C and centrifuged for 20 min at $14,000 \times g$ at 4°C . The supernatants were used to measurement of arginase activity as previously described [20].

Western blotting analysis

Aortic vessels from C57BL/6 mice were lysed in buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, protease inhibitors (Roche co.)) and centrifuged for 30 min at $14,000 \times g$. The amount of protein in the supernatant was determined by the Bradford method. Proteins (100 μg) were loaded on 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad). The blots were incubated with a polyclonal anti-arginase II (Santa Cruz), anti-endothelial nitric oxide synthase (eNOS, BD Bioscience), or anti-actin (Santa Cruz) antibodies followed by the corresponding secondary antibody (Amersham). The signals were detected using an enhanced chemiluminescence detection reagent with X-ray films.

Determination of eNOS dimerization

eNOS dimers and monomers were separated using low-temperature SDS-PAGE and visualized by western blot analysis with specific antiserum as previously described [21]. Band intensities were analyzed by NIH ImageJ Software.

Estimation of NO and ROS generation in isolated mice aorta

We used 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAF-FM) to estimate NO production and dihydroethidine (DHE) to measure ROS production. The change in fluorescence intensity at different time intervals was measured using a microscope as described previously [22].

Aortic vascular tension assay

We measured vascular tension to determine whether increased NO production by KRGE contributes to vasorelaxation in aged mice. Mice were euthanized using isoflurane and the thoracic aorta was rapidly removed. The aorta was placed in ice-cold oxygenated Krebs-Ringer bicarbonate solution (118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 , 1.6 mM $CaCl_2$, 25 mM $NaHCO_3$, 11.1 mM glucose) and cleared of adherent connective tissues. The mouse aorta was cut into 1.5-mm rings and suspended between two wire stirrups (150 μm) in a myograph (Multi myograph system DMT-620) immersed in 10 ml Krebs-ringer (95% O_2 -5% CO_2 , pH 7.4, 37°C). One stirrup was connected to a three-dimensional micromanipulator and the other to a force transducer. The rings were passively stretched at 10-min intervals in 100-mg increments to reach optimal tone (600 mg). After the arterial rings were stretched to their optimal resting tone, the contractile response to 100 mM KCl was determined. For the constriction assay, phenylephrine (PE) was applied at different concentrations ($10^{-9} \sim 10^{-5}$ M), and cumulative responses were recorded. For NO-dependent relaxation responses, vessels were pre-constricted with PE (10^{-6} M) for 15 min, and the endothelium-dependent vasodilator acetylcholine (Ach, $10^{-9} \sim 10^{-5}$ M) was then added to determine relaxation activity. To fur-

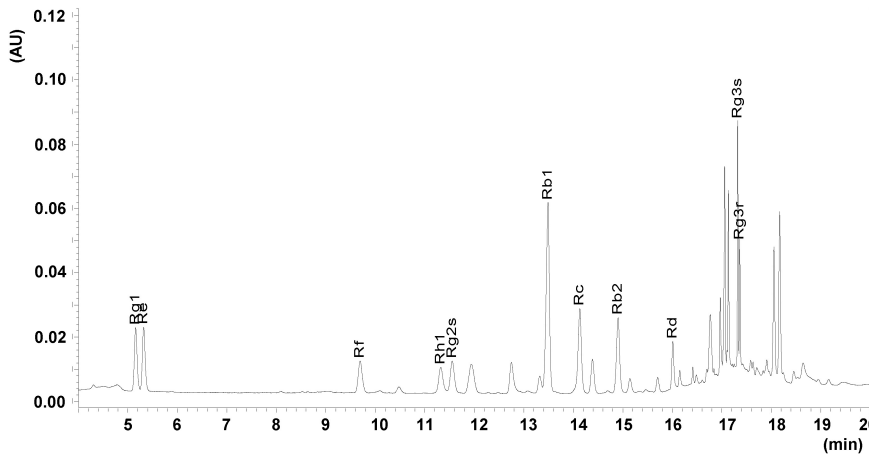


Fig. 1. HPLC analysis of Korean Red Ginseng Extract.

ther confirm the vasorelaxation activity we observed occurred in a NO-dependent manner, we added 1 μ M 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of guanylate cyclase, at the end of the experiments.

Determination of thiobarbituric acid-reactive substances (TBARS)

The precipitate of plasma samples by mixing with trichloroacetic acid (20%) was dispersed in H_2SO_4 (0.05 M). Thiobarbituric acid (TBA, 0.2% in 2 M sodium sulfate) was added and heated for 30 min in a boiling water bath. TBARS products extracted with *n*-butanol and absorbance at 532 nm was measured [23]. Malondialdehyde (MDA) was used as a standard.

Statistical Analysis

All data are represented as mean \pm S.D. of at least four independent experiments. An unpaired Student's *t*-test or 1-way ANOVA was used to assess significant differences. A value of $p < 0.05$ was accepted as significant. Experimental data were analyzed with the GraphPad Prism program (GraphPad Software 4.02).

RESULTS

Effect of KRGE on arginase activity in aortas of aged mice

Mice were orally administered KRGE at 10 or 20 mg/mouse/day for 4 weeks. Arginase activity was significantly higher in the aorta of aged mice than in the young control mice (140.0 ± 13.5 vs. $100 \pm 6.9\%$, $p < 0.01$) (Fig. 2). KRGE doses reduced arginase activity in the aged mice to 117.1 ± 10.2 (10 mg/mouse/day) and 110.0 ± 9.2 (20 mg/mouse/day) ($p < 0.05$).

KRGE enhances NO generation and attenuates ROS production in endothelium of aged mice

Given that arginase competes with nitric oxide synthase (NOS) for L-arginine and negatively regulates NO production, we measured whether KRGE-dependent inhibition

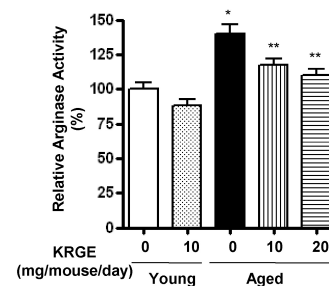


Fig. 2. Increased arginase activity in aged mice aorta was attenuated by Korean Red Ginseng Extract administration. Oral administration of Korean Red Ginseng Extract for 4 weeks resulted in decreased arginase activity in aged mice. * vs. young untreated, $p < 0.01$; ** vs. aged untreated, $p < 0.01$.

of arginase activity was associated with an increase in NO production. As demonstrated in Fig. 3A, NO production was significantly decreased in aged mice compared to young control mice (0.58 ± 0.16 vs. 0.90 ± 0.06 change in DAF fluorescence/sec, $p < 0.01$). KRGE administration restored NO generation to 0.87 ± 0.11 and 0.91 ± 0.14 change in DAF fluorescence/sec in the 10 mg/mouse/day and 20 mg/mouse/day groups, respectively ($p < 0.01$). KRGE also increased NO production in young mice (1.13 ± 0.09 change in DAF fluorescence/sec, $p < 0.01$). ROS generation was significantly higher in aged mice than in their young counterparts (Fig. 3B, 0.52 ± 0.13 vs. 0.31 ± 0.04 change in DHE fluorescence/sec, $p < 0.01$), and was significantly reduced with 10 mg/mouse/day and 20 mg/mouse/day KRGE (0.37 ± 0.13 and 0.28 ± 0.06 change of DHE fluorescence/sec, respectively, $p < 0.01$ compared to untreated aged mice). KRGE also attenuated ROS generation in young mice (0.24 ± 0.02 change in DHE fluorescence/sec, $p < 0.01$). Treatment with the NOS inhibitor L-NAME reduced ROS generation in aged mice (0.29 ± 0.11 change in DHE fluorescence/sec, $p < 0.01$).

KRGE administration enhanced eNOS coupling

Based on the NO production and ROS generation data, we next assessed protein expression to find the underlying mechanism. The eNOS protein expression was significantly higher in aged mice than in young mice (1.23 ± 0.04 vs.

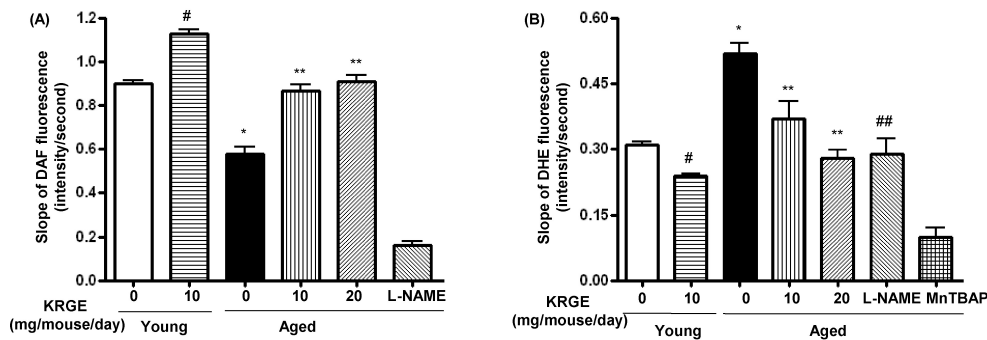


Fig. 3. Korean Red Ginseng Extract restored impaired endothelial function in aged mice. Isolated aortic segments were incubated with 4,5-Diaminofluorescein diacetate (DAF-AM, 5 μ M) and fluorescence was measured in real-time (endothelium side up). The slope of DAF-AM fluorescence was determined. (A) Korean Red Ginseng Extract (KRGE) administration increased the slope of DAF fluorescence in young vessels (# vs. young untreated, $p < 0.01$). The slope of DAF fluorescence was lower in aged mouse aorta (* vs. young untreated, $p < 0.01$) and increased after KRGE ingestion (** vs. aged untreated, $p < 0.01$; $n = 4$ mice). N^G -Nitroarginine Methyl Ester (L-NAME) was used as a control. (B) Reactive Oxygen species (ROS) production in aortic endothelium was measured with dihydroethidium (DHE, 5 μ M), and the slope of DHE fluorescence was determined using cumulative data. KRGE intake decreased ROS production in both young and old mice (# vs. young untreated, $p < 0.01$; * vs. young untreated, $p < 0.01$; ** vs. aged untreated, $p < 0.01$; ## vs. aged untreated, $p < 0.01$; $n = 4$ mice). MnTBAP was used as a control.

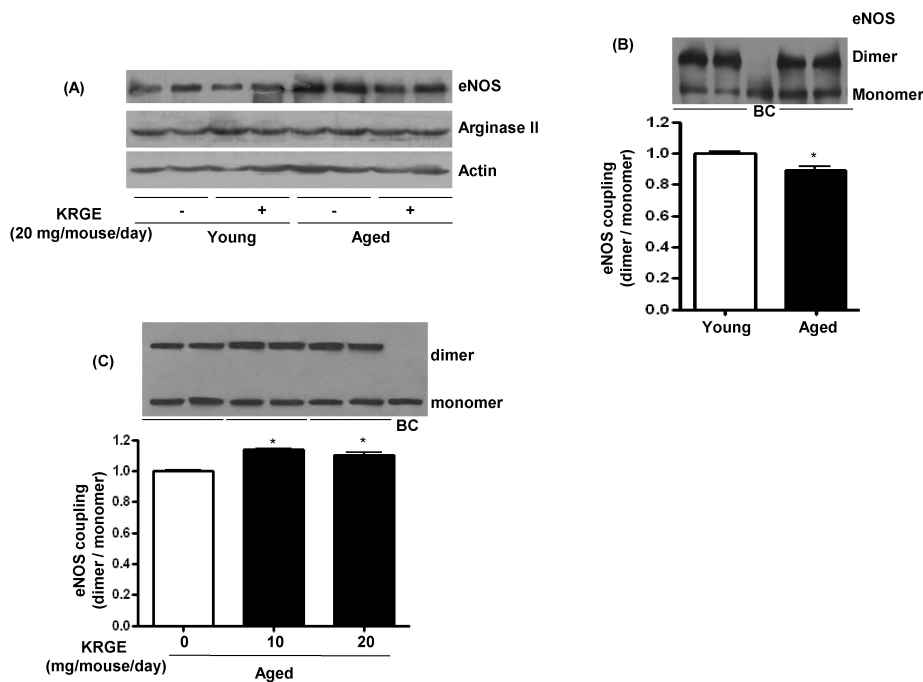


Fig. 4. Korean Red Ginseng Extract improves age-dependent endothelial Nitric Oxide Synthase uncoupling. (A) KRGE did not affect protein expression in the aortas of young or aged mice. (B) Endothelial nitric oxide synthase (eNOS) dimerization was analyzed by low-temperature sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The eNOS was more uncoupled in aged mouse aorta (* vs. young untreated, $p < 0.05$). (C) Korean Red Ginseng Extract (KRGE) increased eNOS coupling in aged mouse aorta (* vs. aged untreated, $p < 0.05$).

1.0 ± 0.03 , $p < 0.01$) and was unaffected by KRGE administration (Fig. 4A). Arginase II protein expression was not different between the groups (Fig. 4A). The eNOS coupling was lower in aged mice than in their young counterparts (Fig. 4B) and was restored with KRGE administration (Fig. 4C). These results imply that endothelial function is impaired through attenuated eNOS coupling despite increased eNOS expression, and that KRGE administration can increase eNOS coupling in the aorta of aged mice.

NO-dependent relaxation of aortic vessels was augmented with KRGE administration

With the above data that KRGE administration induced eNOS coupling and NO production, we measured vascular tension to determine whether increased NO production by KRGE contributes to vasorelaxation in aged mice. The vasorelaxation responses in aged mice were significantly attenuated compared to those from the young (E_{max} $73.30 \pm 2.50\%$ vs. $97.16 \pm 4.08\%$, $p < 0.01$; $-\log EC_{50}$ 6.81 ± 0.09 M vs. 7.23 ± 0.13 M, * $p < 0.01$). KRGE administration to young and aged mice enhanced vasorelaxation responses (** $p < 0.01$)

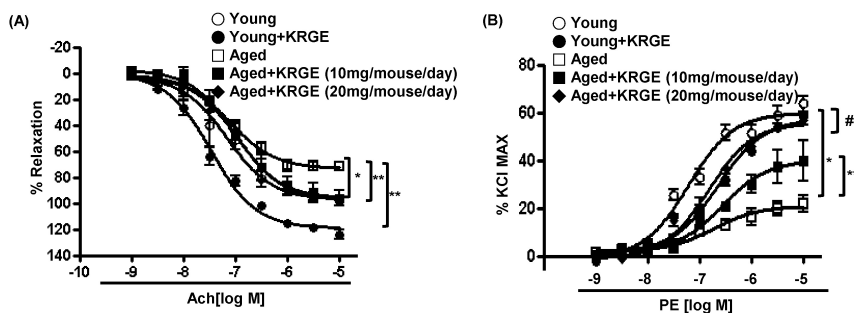


Fig. 5. Korean Red Ginseng Extract improved vascular reactivity impairments in aged mice. (A) Endothelium-dependent relaxation response to acetylcholine (Ach) was impaired in aged aortas (*young vs. aged, $p < 0.01$). Korean Red Ginseng Extract (KRGE) administration improved relaxation (**aged untreated vs. aged+KRGE, $p < 0.05$). (B) Aged aortic vessels had attenuated contractile responses to phenylephrine (PE) compared to young mice (*young vs. aged, $p < 0.01$). KRGE restored the PE-mediated pressor response in aged aortic vessels (**aged untreated vs. aged+KRGE, $p < 0.01$) and attenuated the responses in young aortic vessels ([#]young vs. young+KRGE, $p < 0.05$).

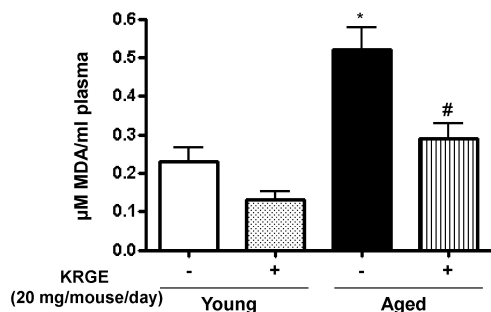


Fig. 6. Korean Red Ginseng Extract administration reduced peroxynitrite formation. Peroxynitrite content in plasma was measured by Thiobarbituric acid reactive substances (TBARS) assay. Aged mice had greater peroxynitrite content than their young counterparts (*young vs. aged, $p < 0.01$; $n = 6$). Korean Red Ginseng Extract (KRGE) reduced peroxynitrite content in aged mice ([#]aged untreated vs. aged+KRGE, $p < 0.01$; $n = 6$).

(Fig. 5A). As shown in Fig. 5B, the responses to PE were markedly attenuated in aortas from aged mice (E_{max} , aged vs. young, $20.93 \pm 1.68\%$ vs. $59.92 \pm 1.83\%$, $p < 0.01$; $-\log EC_{50}$, aged vs. young, 7.72 ± 0.20 M vs. 8.21 ± 0.08 M, * $p < 0.01$). However, KRGE administration enhanced vasoconstrictor responses in aged mice as determined by E_{max} ($40.06 \pm 3.32\%$ and $55.84 \pm 1.54\%$ for 10 mg/mouse/day and 20 mg/mouse/day KRGE, respectively, ** $p < 0.01$ vs. untreated aged), but not $-\log EC_{50}$ was not significant between groups. Interestingly, KRGE attenuated vasoconstriction response to PE in young mice ([#] $p < 0.05$). The response to the endothelium-independent vasodilator sodium nitroprusside (SNP) was not significantly different in any of the groups (data not shown).

KRGE prevents peroxynitrite formation in aged mice

Because oxidative species such as peroxynitrite increase arginase activity [24], and enhanced peroxynitrite formation is associated with vascular aging [25], we measured lipid peroxidation using a TBARS assay. Lipid peroxidation was significantly increased in the plasma of aged mice (0.52 ± 0.13 vs. 0.23 ± 0.08 μM MDA/ml plasma in young mice, $p < 0.01$) (Fig. 6). KRGE administration (20 mg/mouse/

day) reduced lipid peroxidation in aged mice to a level similar to young untreated mice (0.29 ± 0.09 μM MDA/ml plasma, $p < 0.01$ compared to aged untreated mice) (Fig. 6).

DISCUSSION

Endothelial arginase reduces eNOS activity by depleting the critical substrate L-arginine, and this increased arginase activity reduces NO bioavailability and contributes to vascular diseases such as aging, hypertension, and atherosclerosis [7,21,26,27]. Here we showed that oral administration of KRGE for 4 weeks increased NO generation, reduced ROS production by inhibiting arginase activity, induced vasorelaxation in aortic vessels from aged mice, and reduced plasma peroxynitrite formation. These findings suggest KRGE consumption may provide vasoprotective benefits for the aging cardiovascular system.

Ginseng is beneficial in the treatment of various diseases. KRGE eliminates $\text{O}_2^{\cdot-}$ [13], promotes endothelial cell proliferation [9], protects against H_2O_2 -induced cell death by [14], and induces heme oxygenase-1 expression [15] in the vasculature. We demonstrated that KG-WE-dependent arginase inhibition contributed to an increase of NO generation and decrease of ROS production in isolated mice aortas (Fig. 3). These effects resulted in the induction of NO-dependent vasorelaxation (Fig. 5). These results are consistent with previous demonstrations that arginase inhibition accentuates NO release in rat aortic endothelium [7], bovine pulmonary endothelial cells [28], and porcine coronary artery model [29]. The main mechanism of KG-WE-dependent NO release is likely due to increased eNOS coupling resulting from increased L-arginine availability (Fig. 4), in line with previous data [21]. Therefore, these data further support that KRGE can play important role in improving age-related vascular health. The beneficial effects of ginseng on the vascular system are likely complex and may depend on the activation of Akt/PI3K signal transduction [17], inhibition of angiotensin converting enzyme [30], and inhibition of calcium ion influx [31].

Aging is associated with changes in arterial wall structure and function, the most frequent being luminal enlargement, vessel wall thickening due to intimal and medial expansion, elastin depletion and fragmentation, collagen and calcium deposition, glycation of proteins, and impaired vas-

omotor function associated with endothelial dysfunction [32-34]. These alterations increase vascular stiffness, which is an independent risk factor for cardiovascular morbidity and mortality [35-37]. Arginase increases the synthesis of polyamines, which stimulate vascular smooth muscle and endothelial cell proliferation, and L-proline, which increases collagen deposition. Together, these cellular alterations contribute to endothelial dysfunction and arterial stiffening associated with aging. Although increased arginase activities were well associated with endothelial dysfunction in diseases-related animal models [7,28,29], arginase itself currently had a limitation of usefulness to extent the human in terms of aging.

Vascular ROS production is enhanced in aged blood vessels [4,25,38,39], and the enzymatic system eNOS may contribute to increased ROS production in pathophysiological states. Typically, eNOS produces the vasoprotective molecule NO. However, it can also produce $O_2^{\cdot-}$ in the absence of substrate, L-arginine, or cofactor, BH_4 , because electrons flow from the reductase domain in the heme to molecular oxygen rather than to L-arginine in uncoupled eNOS. We showed increased eNOS uncoupling (Fig. 4B), arginase activity (Fig. 2), and peroxynitrite formation (Fig. 6) in aged vessels. Furthermore, preincubation of L-NAME, the eNOS inhibitor, attenuated ROS production on the endothelium of aged mice (Fig. 3B), which is consistent with previous observations [40]. Peroxynitrite may stimulate arginase II activation via ROCK-dependent pathway without a change in mRNA and protein levels [24]. Peroxynitrite reacts also with the redox-active cysteine (Cys¹⁸) of RhoA, which results in enhanced release of GDP from RhoA and thus modulates its activity [4]. Indeed, we found plasma peroxynitrite formation, as measured by the TBARS assay, was significantly higher in aged mice compared to their young counterparts (Fig. 6), which is consistent with previous research [25].

The promoter region of the eNOS gene has binding site to a shear stress-responsive element [41] indicates that shear stress and hemodynamic forces may increase eNOS expression [42,43]. Consistent with these findings, eNOS expression is higher in the aorta of aged animals [44], but is not changed in the artery [45] and is decreased in the arteriole [3]. Increased eNOS protein expression in the aged aorta may be a mechanism to compensatory the endothelial dysfunction associated with increased arginase activity (Fig. 4A).

The expression of inducible nitric oxide synthase (iNOS) has been reported in old rat model [46]. Therefore, increased iNOS expression and/or activity may be an additional possible mechanism to explain the decreased contraction in the aortic rings of aged mice in response to PE (Fig. 5B). Although we could not detect the expression of iNOS protein at aortic vessels from aged mice in the presence or absence of KRGE (data not shown), we could not exclude the involvement of iNOS.

In summary, we demonstrate that KRGE inhibits the age-associated increase in arginase activity in the aorta, and is associated with enhanced eNOS dimerization and increased NO production. Furthermore, KRGE augments vasorelaxation in a NO-dependent manner and attenuates peroxynitrite formation. These findings suggest that KRGE possesses therapeutic potential for cardiovascular diseases associated with aging.

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