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Research article

Rg3-enriched Korean Red Ginseng extract inhibits blood-brain barrier disruption in an animal model of multiple sclerosis by modulating expression of NADPH oxidase 2 and 4

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ABSTRACT

Background: Multiple sclerosis (MS) and its animal model, the experimental autoimmune encephalomyelitis (EAE), are primarily characterized as dysfunction of the blood-brain barrier (BBB). Ginsenoside-Rg3-enriched Korean Red Ginseng extract (Rg3-KRGE) is known to exert neuroprotective, anti-inflammatory, and anti-oxidative effects on neurological disorders. However, effects of Rg3-KRGE in EAE remain unclear.

Methods: Here, we investigated whether Rg3-KRGE may improve the symptoms and pathological features of myelin oligodendroglial glycoprotein $(MOG)_{35-55}$ peptide – induced chronic EAE mice through improving the integrity of the BBB.

Results: Rg3-KRGE decreased EAE score and spinal demyelination. Rg3-KRGE inhibited Evan's blue dye leakage in spinal cord, suppressed increases of adhesion molecule platelet endothelial cell adhesion molecule-1, extracellular matrix proteins fibronection, and matrix metallopeptidase-9, and prevented decreases of tight junction proteins zonula occludens-1, claudin-3, and claudin-5 in spinal cord following EAE induction. Rg3-KRGE repressed increases of proinflammatory transcripts cyclooxygenase-2, inducible nitric oxide synthase, interleukin (IL)-1 beta, IL-6, and tumor necrosis factor-alpha, but enhanced expression levels of anti-inflammatory transcripts arginase-1 and IL-10 in the spinal cord following EAE induction. Rg3-KRGE inhibited the expression of oxidative stress markers (MitoSOX and 4-hydroxynonenal), the enhancement of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) and NOX4, and NADPH activity in the spinal cord of chronic EAE mice. Furthermore, apocynin, a NOX inhibitor, mimicked beneficial effects of Rg3-KRGE in chronic EAE mice.

Conclusion: Our findings suggest that Rg3-KRGE might alleviate behavioral symptoms and pathological features of MS by improving BBB integrity through modulation of NOX2/4 expression.

1. Introduction

Multiple sclerosis (MS) is a spontaneous, acquired, inflammatory demyelinating disease of the human central nervous system (CNS) that can appear as a variable course of neurological disabilities [1,2]. Primarily pathogenesis of MS is characterized by complex interaction between CNS and immune system, two of the most complicated biological systems, through the blood-brain barrier

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(BBB) [1,2]. Experimental autoimmune encephalomyelitis (EAE) is widely used as an *in vivo* model of MS to study its behavioral symptoms such as paralysis, immunological events such as migration/infiltration of resident microglia and peripheral immune cells, and histopathological features such as neurodegeneration including demyelination in brain, spinal cord, and optic nerve [3,4]. In both MS and EAE, the activation of auto-aggressive immune cells can penetrate the BBB and significantly contribute to neuroinflammation, demyelination, and neuronal damage. The BBB is composed of endothelial cells, pericytes, perivascular astrocytes, and a number of cellular and molecular structures between these cells. The BBB relies on the interendothelial tight junctions that are present between endothelial cells of the CNS capillaries to provide a closed environment for the CNS [5–7]. Tight junctions, also known as occluding junctions or zonulae occludens, are multiprotein junctional complexes that can prevent leakage of transported solutes and water and seal the paracellular pathway [5–7].

Reactive oxygen species (ROS) produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases play a pivotal role in various events underlying MS and EAE pathology [8]. In the early phase of lesion formation, ROS are reported to induce dysfunction of BBB and mediate transendothelial migration of peripheral immune cells such as T cells and macrophages. ROS also contribute to lesion persistence in MS and EAE by degradation and phagocytosis of myelin and induction of axonal and oligodendrocyte damage [9–11]. To escape harmful activities of ROS, antioxidants such as vitamins (ascorbic acid, retinol, beta-carotene, and alpha tocopherol) may be used to provide positive effects for MS patients, although they have limitation in both cellular migration/infiltration and lesion progression [12,13]. Antioxidants such as Korean ginseng (Panax ginseng Meyer) [14,15], flavonoids [9], and catalase [13] have been reported to have beneficial effects on EAE. These ROS scavengers can affect cellular migration/infiltration through BBB, axonal damage, and demyelination, leading to mitigated clinical symptoms [12,13].

Korean ginseng has been taken as a herbal remedy. It has been lauded as the “elixir of life” for over two thousand years around the world [16,17]. Korean Red Ginseng (KRG) is a steamed and dried Korean ginseng with multifunctional efficacy, including promotion of physical strength and immunity [16,17]. Major active constituents of KRG are ginsenosides that can be divided into three groups: the panaxadiol group represented by Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, and Rs1, the panaxatriol group consisting of Re, Rf, Rg1, Rg2, Rh1, and compound K, and the oleic acid group consisting of Ro. Ginsenosides such as Rg2, Rg3, Rg5, Rh1, Rh2, Rk1, and compound K are called as representative KRG-specific ginsenosides. Their contents are increased after the steaming process [18]. They show a variety of pharmacodynamics in biological systems including nervous system [15–17,19]. Specifically, ginsenoside-Rg3 is not naturally present in Korean ginseng [20,21]. It is produced by the loss of the glucose group linked with the C-20 of ginsenoside Rc, Rd, Rb1, or Rb2 when Korean ginseng is heated to a high temperature [22].

Ginsenoside-Rg3 is known to have positive effects on neuronal apoptosis, neuroinflammation [23], cerebral ischemia, and myocardial infarction [24] in rat, lipopolysaccharide-induced acute lung injury in mice [25], and mast cell-mediated allergic inflammation [26]. Interestingly, ginsenoside-Rg3 can improve endothelial dysfunction from oxidative stress by upregulating nuclear factor E2-related factor 2 (Nrf2)-antioxidant responsive element (ARE) pathway through activation of Akt in adriamycin-induced cardiotoxicity model [27]. It can also inhibit lipopolysaccharide-induced adhesion molecule expression in human umbilical vein endothelial cell and C57BL/6 mice [28]. For reasons given above, ginsenoside-Rg3-enriched KRGE (Rg3-KRGE) has been studied extensively, demonstrating possible activities such as vasodilating [29], anti-inflammatory [29], and antioxidant properties [30]. Interestingly, Rg3-KRGE can improve vascular function in spontaneously hypertensive rats by increasing endothelial nitric oxide synthase (eNOS) phosphorylation and nitric oxide (NO) production or inhibiting intercellular adhesion molecule (ICAM)-1 and cyclooxygenase-2 expressions in endothelial cells [31,32]. Meanwhile, ginsenoside-Rg3 can enhance the expression of antioxidant enzymes and inhibit the expression of inflammatory cytokines in chronic intermittent heat stress-induced testicular damage [30]. These findings indicate that Rg3-KRGE may be able to prevent BBB disruption in neurological disorders. Since the integrity of the BBB might be damaged in chronic autoimmune inflammatory disorder and result in its dysfunction [7], the objective of the current study is to explore whether anti-inflammatory and antioxidant activities of Rg3-KRGE might improve BBB integrity in chronic EAE model.

2. Materials and methods

2.1. Experimental animals and ethic approval

Eight-week-old female C57BL/6NTac mice (18–20g) were purchased from Narabiotec Co., Ltd. (Pyeongtaek, Republic of Korea). The mice were housed in a 12 light/12 dark cycle (light on 08:00 to 20:00) with room temperature at 23 ± 4°C and relative humidity of 50% ± 10%, and fed food and water *ad libitum*. The animals were allowed to habituate to the housing facilities for 1 week before the experiments. All experimental procedures such was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Kyung Hee University (KHUASP(SE)-17-065). Proper randomization of laboratory animals and handling of data were accomplished in a blinded manner in agreement with recent recommendations from a National Institutes of Health Workshop on preclinical models of neurological disorders [33].

2.2. Experimental group, chronic EAE induction, and behavioral assessment

The experimental group was divided into the following groups (n = 5 per group): The Sham group [vehicle treatment, s.c. + saline, p.o.], EAE [200μg of MOG_{35-55}, s.c. + saline, p.o.], EAE + Rg3-KRGE group [200μg of MOG_{35-55}, s.c. + 37.5, 75, and 150mg/kg of Rg3-KRGE, p.o.], and Rg3-KRGE alone group [vehicle treatment, s.c. + 150mg/kg of Rg3-KRGE, p.o.]. EAE induction was performed according to the published method [14]. Behavioral score was measured daily by a blinder researcher after EAE induction according to the published method [14,34–36]: grade 0, absence of symptoms; grade 1, partial loss of tail tonus; grade 2, paralysis of tail; grade 3, paraparesis; grade 4, paraplegia; grade 5, tetraparesis; grade 6, tetraplegia; and grade 7, death.

2.3. Rg3-KRGE ant its administration

Rg3-KRGE was provided by Korea Society of Ginseng (Seoul, Republic of Korea). KRGE was prepared from the roots of 6-year-old fresh ginseng, *P. ginseng* Meyer, harvested in Republic of Korea by the Korea Ginseng Corporation (Daejeon, Korea). Briefly, KRG was made by steaming fresh ginseng roots at 90–100°C for 3 hours and then drying at 50–80°C. Rg3-KRGE was prepared from KRG extract, which was extracted in seven 12 hours cycles of circulating hot water (87°C) and two 3 hours cycles of circulating hot ethanol (65°C). The extract was concentrated at 50–60°C and spray-dried. Rg3-KRGE contained enriched ginsenoside-Rg3 [Rg3s (33.73mg/g) + Rg3r (6.38mg/g) = 40.11mg/g], major ginsenosides Rb1 (2.90mg/g), Rb2 (1.97mg/g), Rc (0.97mg/g), Rd (1.37mg/g), Re (0mg/g), Rf (1.05mg/g), Rg1 (0mg/g), Rg2s (2.94mg/g), and Rh1 (2.94mg/

g), and other minor ginsenosides, as determined by high-performance liquid chromatography (Korea Society of Ginseng). All administrations started from 7 days before immunization. Rg3-KRGE was dissolved in physiologic saline and administered orally once daily for experimental period with 37.5, 75, and 150mg/kg body weight.

2.4. Histopathological evaluation

At the peak stage (18–20 days) of behavioral symptoms following EAE induction, the sections (10-μm thick) from lumber spinal cords (n = 3 per group) were prepared by previous described [14,34–36]. The sections (n = 3 per spinal cord) were stained with luxol fast blue dye (LFB), dehydrated, and coverslipped. The level of demyelination following LFB staining was measured as previously described [35]: 0, no demyelination; 1, little demyelination, only around infiltrates and involving less than 25% of the white matter; 2, demyelination involving less than 50% of the white matter; 3, diffuse and widespread demyelination involving more than 50% of the white matter.

2.5. Assessment of BBB disruption

On day 18–20 post immunization, the level of BBB disruption was assessed using Evan's blue dye (Sigma-Aldrich) as described previously [35]. Evan's blue staining and immunofluorescent staining of PECAM-1 were performed to detect albumin extravasation (BBB permeability) and BBB disruption, respectively, as previously described [35].

2.6. Immunoblot analysis

At the peak stage (18–20 days) of behavioral symptoms following EAE induction, the lumbar segments of the spinal cords (n = 3 per group) were cropped. Immunoblot analysis was accomplished as previously described [35,37] using mouse anti-platelet endothelial cell adhesion molecule [PECAM-1 (CD31); 1: 500; Santa Cruz biotechnology, Santa Cruz, CA, USA)]. Rabbit anti-fibronectin (1: 500; Santa Cruz biotechnology), Rabbit anti-MMP-9 (1:500, Abcam, Cambridge, UK), rabbit anti-p-ERK/p-JNK/p-P38 (1:1,000; Cell Signaling Technology, Beverly, MA, USA) antibodies, and HRP-conjugated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:1,000; Cell Signaling Technology) was used as normalization for relative protein quantification.

2.7. Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR analysis

At the peak stage (18–20 days) of neurological impairment, the mice used for RT-PCR and real time PCR (n = 3 per group) were anesthetized. Each lumbar spinal cord was removed and deep-frozen. Total RNA was extracted from spinal cord using TRIsure reagent according to the manufacturer's instructions (Bioline, UK). cDNA was synthesized as previous described [14,15] and RT-PCR analysis was performed according to the manufacturer's instructions (RT-PCR kit; Roche, Germany). Real-time RT-PCR was performed using SYBR Green PCR Master Mix (ABI, Warrington, UK) as described previously [14,37,38]. To determine the relative quantification of target gene expression, fold-induction was calculated using the 2-ΔΔCT method, as previously described [39]. All real-time-PCR experiments were performed at least thrice and the mean ± SEM values are presented unless otherwise noted. GABDH was used as a housekeeping endogenous control gene. Sequences of oligonucleotide primers are shown in Supplementary Table 1.

2.8. NADPH oxidase activity evaluation

At the peak stage (18–20 days) of neurological impairment, NADPH oxidase activity was measured using a colorimetric NADP/NADPH assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Lumber spinal cords (n = 3 per group) were lysed in an assay buffer provided in the kit. The lysates were deproteinized by passing through a 10 kD Spin column (Abcam). The assay was performed in a 96-well plate and absorbance was measured with a microplate luminometer (Molecular devices, VERSAmax™ Tunable Microplate Reader San Jose, CA, USA) at 450nm. For every sample, NADPH oxidase activity was calculated as the difference between activities obtained in the presence and absence of NADPH.

2.9. Analysis of MitoSOX activity

MitoSOX activity was measured as previously described [40]. Briefly, free-floating sections (n = 3 per spinal cord) from each group (n = 3 per group) were incubated for 10 minutes to allow the probe to enter the cell and start the reaction within the mitochondria at 37°C in 0.5ml of measurement buffer containing 5mM MitoSOX Red (Molecular Probes, Eugene, OR, USA). Subsequently, these sections were washed twice with PBS and coverslipped.

2.10. Lipopolysaccharide-induced bEND.3 cell culture

The bEnd.3 cell line as a BBB model was obtained from American Type Culture Collection (VA, USA). These cells were cultured in Dulbecco's modified eagle medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a 5% CO_2 incubator. Briefly, cells were seeded into 6-well plates at a density of 5×10^5 cells/well. After 18 hours of culture, cells were treated with ginsenoside-Rg3 at various concentrations (1, 10, and 100μg/ml) at 1 hour before stimulation with lipopolysaccharide (0127:B8; 1μg/ml; Sigma-Aldrich). Four hours after lipopolysaccharide treatment, cells were collected for real time PCR analysis.

2.11. Statistical analysis

Statistical analysis was performed using the SPSS 21.0 package (SPSS Inc, Chicago, USA) for Windows. Neurological scores obtained by EAE induction were analyzed using post-hoc analysis for two-way analysis of variance (ANOVA) with repeated measures with one within-subjects factor (time) and two between-subject factors (Sham and EAE group; EAE and EAE + Rg3-KRGE group). The data from immunofluorescent staining, immunoblotting, and PCR analysis were analyzed using one-way ANOVA with Tukey post hoc test for comparison of multiple groups. The data were presented as mean ± SEM. P values of less than 0.05 were accepted as statistically significant.

3. Results

3.1. Rg3-KRGE alleviates behavioral severity and spinal demyelination of chronic EAE

We first investigated whether 37.5, 75, or 150mg/kg Rg3-KRGE could exert beneficial effects on behavioral symptoms of chronic EAE mice. Mice in the chronic EAE group showed typical behavioral disorders including limp tail and hindlimb paralysis of chronic EAE

as evidenced by ascending paralysis that began at 7–8 days after EAE induction, peaked at 16–18 days, and then persisted until the end of the experiment (Fig. 1A). The 150mg/kg Rg3-KRGE Rg3-KRGE significantly mitigated daily behavioral symptom (Fig. 1A), sum of score (Fig. 1B), and maximum behavioral scores (Fig. 1C and D) of chronic EAE during 12–20 days of the experimental period. Since administration with 150mg/kg Rg3-KRGE was the best effective one in inhibiting EAE symptoms, this dose was used in further studies. Spinal demyelination is a typical histopathological feature of MS patients [3,4]. Thus, we examined the correlation between behavioral score of EAE and demyelination. Levels of spinal demyelination (pale portion) by staining with LFB dye were the highest in spinal white matters of mice in the EAE group on day 18–20 after EAE induction while their levels were significantly lower in spinal cords of 150mg/kg Rg3-KRGE-administered EAE mice (Fig. 1E and F).

3.2. Rg3-KRGE protects BBB integrity and alleviates junctional proteins in spinal cords of chronic EAE mice

We next investigated extravasation level of Evan's blue dye in the spinal cord of EAE mice to determine the effect of Rg3-KRGE on BBB integrity. Evan's blue leakage into spinal cord was observed in mice of the EAE group on days 18–20 after EAE induction (Fig. 2A). According to quantitative results, lower levels of Evan's blue leakage were observed in spinal cords of 150mg/kg Rg3-KRGE-administered EAE mice compared with those in mice of the EAE group (Fig. 2B). Tight junctions can act as pivotal components of the BBB, create a paracellular barrier between endothelial cells, and protect CNS from the external environment. It has been previously reported that normal distributions and structures of tight junctions are disrupted in CNS tissues of MS patients and EAE mice [5–7]. To further investigate positive effects of Rg3-KRGE on disruption of the BBB basement membrane after EAE induction, we examined expression levels of transmembrane junctional molecules on day 18–20 following EAE induction using real-time PCR analysis (Fig. 2C–E). Results showed that mRNA expression levels of zonula occludens-1, claudin-3, and claudin-5 were significantly reduced in spinal cords of EAE mice compared to those in sham group of mice. However, administration with 150mg/kg Rg3-KRGE induced distinguished enhancements of zonula occludens-1, claudin-3, and claudin-5 (Fig. 2C–E).

3.3. Rg3-KRGE enhances protein expression levels of PECAM-1 and fibronectin in spinal cords of chronic EAE mice

PECAM-1 and fibronectin are representative indicators of BBB disruption [41,42]. Thus, their expression levels in spinal cords were investigated by immunoblot. PECAM-1 and fibronectin levels were significantly enhanced in spinal cords of chronic EAE mice. Protein levels of these two factors were repressed in the group administered with 150mg/kg Rg3-KRGE compared to those in the EAE group (Fig. 3A and B). These results indicated that Rg3-KRGE blocked the upregulation of both PECAM-1 and fibronectin in spinal cords of EAE mice, consistent with the pattern of co-staining of Evan's blue and PECAM-1/or fibronectin (Fig. 3D). It is known that gelatinase B (matrix metallopeptidase 9; MMP-9) deficiency can shelter endothelial PECAM-1 expression [43] while fibronectin upregulates gelatinase B [44]. Thus, their expression levels in spinal cords were performed by immunoblot. Protein levels of MMP-9 in spinal cords of EAE mice were significantly inhibited after administration with 150mg/kg Rg3-KRGE compared to those in mice of the EAE group (Fig. 3C).

3.4. Rg3-KRGE suppresses inflammatory response in spinal cords of chronic EAE mice

Loss of BBB integrity in CNS inflammatory responses triggered by infection and autoimmunity is generally related to the development of neurological signs [45]. Thus, we investigated the level of inflammatory responses to determine possible mechanisms underlying the positive effects of Rg3-KRGE (Fig. 4A–E). RT-PCR

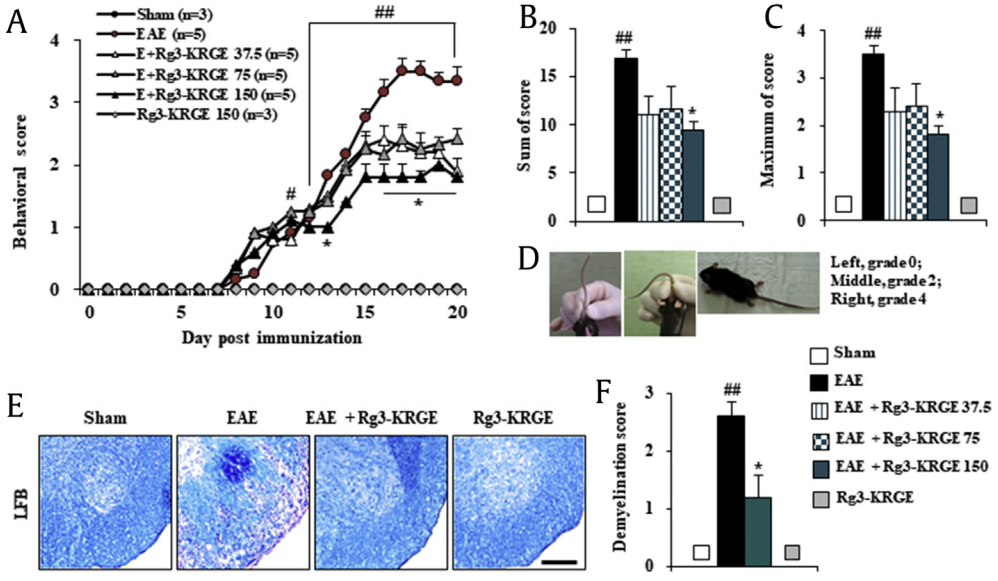

Fig. 1. Administration with Rg3-KRGE alleviates behavioral severity and spinal demyelination of chronic EAE mice. (A-D) For 20 days after EAE induction, neurological impairment was accessed once daily (n = 5 per group). (A) Mean daily behavioral scores of Rg3-KRGE-administrated mice at different doses (37.5, 75, and 150mg/kg) for 20 days following EAE induction. Mean behavioral score was assessed from day 1 until day 20 following EAE induction (B). The maximum behavioral score from each mouse during the entire observation period was recorded (C). Representative photo for behavioral score 0, 2, and 4 (D). **(E and F)** At 18–20 days after EAE induction, spinal cord sections (n = 3 per spinal cord) were obtained from all groups (n = 3 per group), stained with LFB (E), and quantified (F). Scale bar = 100μm. Experiments were repeated three times. Data are presented as means ± SE. ANOVA test; #P < 0.05 and ##P < 0.01 vs. Sham group; *P < 0.05 and **P < 0.01 vs. EAE group; N.D., not detected.

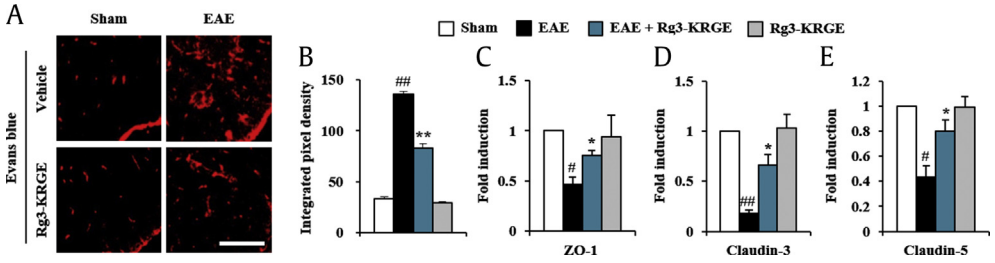

Fig. 2. Rg3-KRGE administration protects BBB integrity and ameliorates loss of tight junction proteins in chronic EAE mice. (A and B) At 18–20 days after EAE induction, sterilized Evan's blue solution was intravenously injected to each mouse. Lumbar spinal cords (n = 3 per group) were immediately removed and sectioned and images were captured (n = 3 per group). (A) Representative images of spinal cord sections with Evan's blue staining in experimental groups. Scale bar = 100μm. (B) Quantification of the percentage of Evan's blue-positive area comparing the entire spinal section (n = 3 per mouse). **(C-E)** At 18–20 days after EAE induction, lumbar spinal cords (n = 3 per group) from each group (n = 3 per group) were analyzed by real-time PCR to investigate mRNA expression levels of zonula occludens-1 (C), claudin-3 (D), and claudin-5 (E). RNA level of each target gene was normalized to that of GAPDH mRNA. Experiments were repeated three times. Data are presented as means ± SE. ANOVA test; #P < 0.05 and ##P < 0.01 vs. Sham group; *P < 0.05 and **P < 0.01 vs. EAE group.

was used to measure mRNA expression levels of pro-inflammatory enzymes COX-2 and iNOS and pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in spinal cords. Markedly elevated mRNA expression levels of COX-2, iNOS, IL-1β, IL-6, and TNF-α were detected in spinal cords of chronic EAE mice. They were significantly inhibited by 150mg/kg Rg3-KRGE administration (Fig. 4A–E). Meanwhile, mRNAs of anti-inflammatory enzyme arginase-1 and anti-inflammatory cytokine IL-10 were slightly upregulated in EAE mice, but greatly upregulated in EAE mice after administration with 150mg/kg Rg3-KRGE (Fig. 4F and G). Since activation of MAPKs is an important mechanism in inflammatory response and up-regulation of iNOS and COX-2 during inflammation is controlled by the activation of pro-inflammatory transcription factor MAPKs, we investigated protein expression levels of p-ERK, p-JNK, and p-p38 in spinal cords by immunoblot. Significantly elevated mRNA expression levels of p-ERK, p-JNK, and p-p38 in spinal cords of chronic EAE mice were detected. Their expression levels were then markedly inhibited by administration with 150mg/kg Rg3-KRGE (Fig. 4H–J).

3.5. Rg3-KRGE suppresses NADPH oxidase activity in spinal cords of chronic EAE mice

Since oxidative stress is increasingly implicated as a co-factor of tissue injury in inflammatory/demyelinating disorders of the CNS such as MS [46], we also investigated whether oxidative stress was altered by Rg3-KRGE administration in chronic EAE mice. Intensities of MitoSOX, a mitochondrial superoxide indicator, were increased in spinal cords of chronic EAE mice (61.4%) compared to those in the sham group (0.8%), while the enhancement in the intensity was prevented (18.5%) by 150mg/kg Rg3-KRGE administration (Fig. 5A and B). Moreover, protein expression levels of 4-HNE, one of major end products of lipid peroxidation, were upregulated in spinal cords of chronic EAE mice (96.2%) than those in the sham group (35.8%), while such upregulation was inhibited (57.1%) by 150mg/kg Rg3-KRGE administration (Fig. 5C). Oxidation status in the spinal cord was determined based on NADPH oxidase (NOX) expression and NADPH activity [47,48]. EAE mice showed overexpressed mRNA levels of NOX1, NOX2, and NOX4 (Fig. 5D–F). Interestingly, administration with 150mg/kg Rg3-KRGE did not significantly affect mRNA expression of NOX1 compared to control (sham group) (Fig. 5D). However, enhanced mRNA expression levels of NOX2 and NOX4 by EAE induction were significantly inhibited by administration with 150mg/kg Rg3-KRGE (Fig. 5E and F). Upregulated NADPH activity by EAE induction was also significantly inhibited by administration with 150mg/kg Rg3-KRGE (Fig. 5G). According to Figs. 1–5, 150mg/kg Rg3-KRGE can inhibit BBB disruption in an animal model of MS by modulating the expression of NADPH oxidase 2 and 4. Thus, we additionally investigated whether ginsenoside Rg3 might have significant effects on BBB protection by altering levels of NOX2 and NOX4. In the lipopolysaccharide-induced bEND.3 cell line, mRNA levels of NOX2 and NOX4 were overexpressed. However, pretreatment with ginsenoside Rg3 significantly prevented such enhancement in their expression (Fig. 5H and I).

3.6. NOX inhibitor mimics beneficial effects of Rg3-KRGE in chronic EAE mice

Finally, to corroborate that Rg3-KRGE could alleviate behavioral symptoms and demyelination of EAE by improving BBB integrity through modulation of NOX2/4 expression (Figs. 1–5), we investigated whether apocynin, a NOX inhibitor, might mimic beneficial

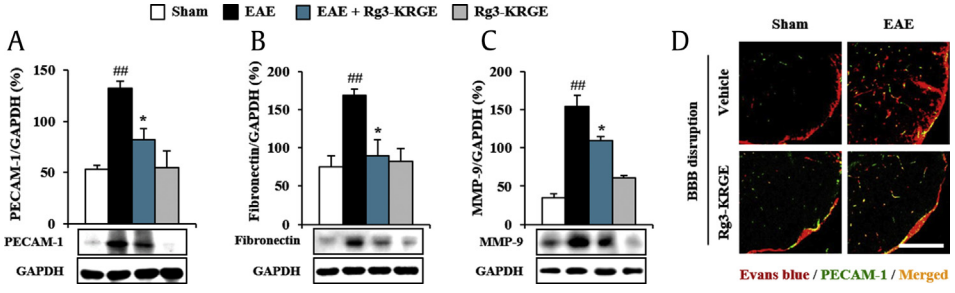

Fig. 3. Rg3-KRGE enhances protein expressions of PECAM-1 and fibronectin in spinal cords of chronic EAE mice. (A-C) At 18–20 days after EAE induction, lumbar spinal cords from each group (n = 3 per group) were analyzed by immunoblot to investigate protein expression levels of PECAM-1 (A), fibronectin (B), and MMP-9 (C). Protein expression level of each target was normalized to that of GAPDH protein. **(D)** At 18–20 days after EAE induction, lumbar spinal cords from each group (n = 3 per group) were analyzed by double immunofluorescence staining to investigate the level of co-staining of Evan's blue and PECAM-1/or fibronectin. Scale bar = 100μm. Experiments were repeated three times. Data are presented as means ± SE. ANOVA test; #P < 0.05 and ##P < 0.01 vs. Sham group; *P < 0.05 and **P < 0.01 vs. EAE group.

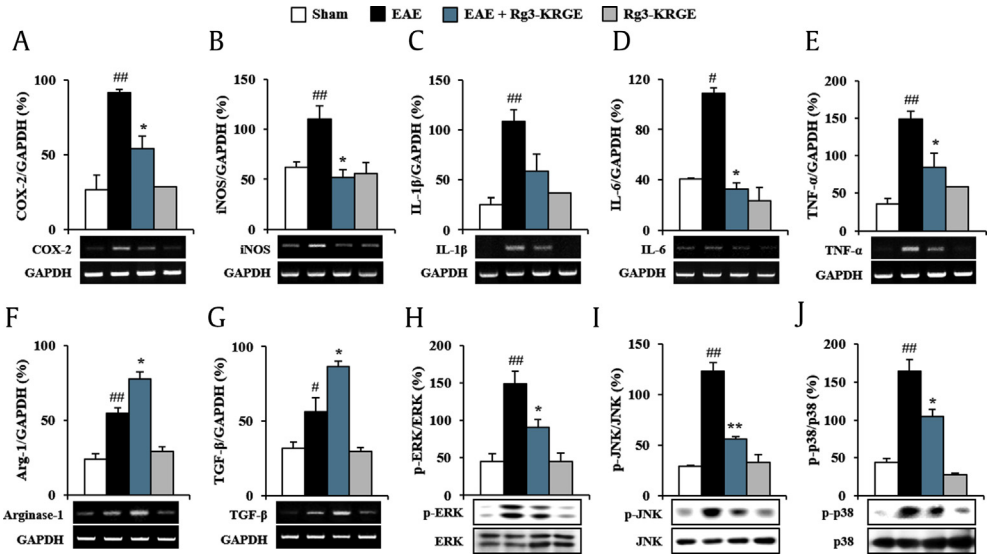

Fig. 4. Rg3-KRGE suppresses inflammatory response in spinal cords of chronic EAE mice. (A-G) At 18–20 days after EAE induction, lumbar spinal cords from each group (n = 3 per group) were analyzed by RT-PCR to investigate mRNA expression levels of COX-2 (A), iNOS (B), IL-1β (C), IL-6 (D), TNF-α (E), Arginase-1 (F), and IL-10 (G). **(H-J)** At 18–20 days after EAE induction, lumbar spinal cords from each group (n = 3 per group) were analyzed by double immunofluorescence staining to investigate protein levels of p-ERK (H), p-JNK (I), and p-p38 (J). Protein level of each target was normalized to that of GAPDH protein. Experiments were repeated three times. Data are presented as means ± SE. ANOVA test; #P < 0.05 and ##P < 0.01 vs. Sham group; *P < 0.05 and **P < 0.01 vs. EAE group; n.s., not significant.

effects of Rg3-KRGE in chronic EAE mice (Fig. 6). Interestingly, apocynin (15 and/or 30mg/kg body weight, i.p.) reduced EAE score in daily course and sum of score (Fig. 6A and B) and the level of demyelination by LFB staining (Fig. 6C) and Western bolt for MBP (Fig. 6H) in spinal cords of chronic EAE mice. It also caused reduction in cellular infiltration to lesion (Fig. 6D). Furthermore, apocynin inhibited the increase in immunoreactivity or protein expression levels of BBB-associated components (GFAP, PECAM-1, and MMP-9) (Fig. 6E, F, and I-K), representative inflammatory molecules (p-ERK, p-JNK, and p-p38) (Fig. 6L–N), and representative oxidative stress markers (MitoSOX and 4-HNE) (Fig. 6G and O) in spinal cords of chronic EAE mice.

4. Discussion

EAE is a widely-used rodent model of MS characterized by T cell-mediated inflammation, BBB disruption, demyelination, and axonal injury [3,4]. BBB dysfunction is one of pivotal features in the pathology of MS and EAE [3,4]. Peripheral inflammatory/immune cells and toxic factors are recruited and infiltrated into the CNS through disrupted BBB, resulting in neural degeneration [3,4]. Thus, agents that can maintain BBB integrity may have potential to treat many neurological disorders, including Alzheimer's disease and MS [49]. Here, we examined the potential positive effect of Rg3-KRGE on behavioral symptoms, spinal demyelination, BBB disruption,

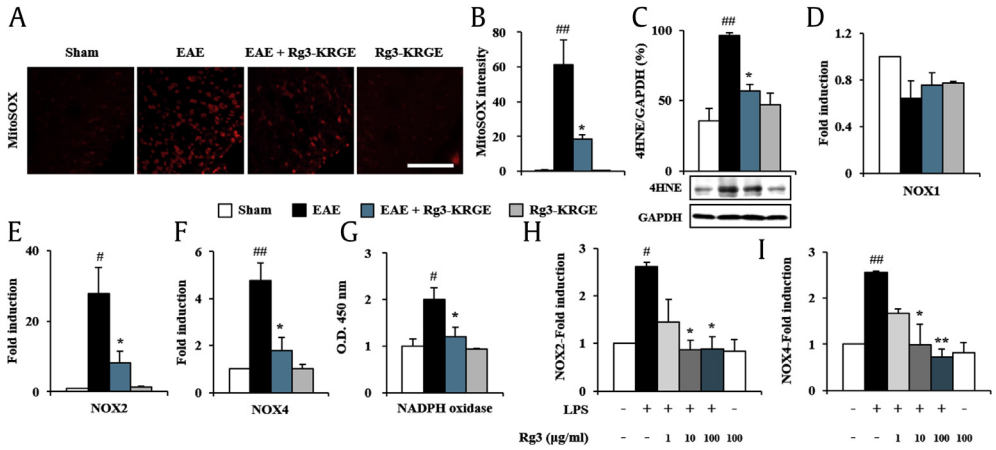

Fig. 5. Rg3-KRGE suppresses NADPH oxidase activity in spinal cords of chronic EAE mice. (A-C) At 18–20 days after EAE induction, lumbar spinal cords from each group (n = 3 per group) were analyzed by MitoSOX and immunoblot analyses to investigate expression levels of oxidative stress markers mitochondrial superoxide (A and B) and 4-HNE (C), respectively. Scale bar = 100μm. Protein expression level of 4-HNE was normalized to that of GAPDH protein. **(D-G)** At 18–20 days after EAE induction, lumbar spinal cords from each group (n = 3 per group) were analyzed by real-time PCR to investigate mRNA expression levels of NOX1 (A), NOX-2 (B), and NOX4 (C) and by colorimetric assay to measure NADPH oxidase activity (D). The mRNA expression level of each target gene was normalized to that of GAPDH mRNA and NADPH oxidase activity was normalized to that of sham group. **(H and I)** One hour after treatment with ginsenoside Rg3 in bEND.3 cells, these cells were stimulated with lipopolysaccharide. Cells from each group (n = 3 per group) were analyzed by real-time PCR to investigate mRNA expression levels of NOX2 (H) and NOX4 (I). The mRNA level of each target gene was normalized to that of GAPDH mRNA. Experiments were repeated three times. Data are presented as means ± SE. ANOVA test; #P < 0.05 and ##P < 0.01 vs. Sham group; *P < 0.05 and **P < 0.01 vs. EAE group.

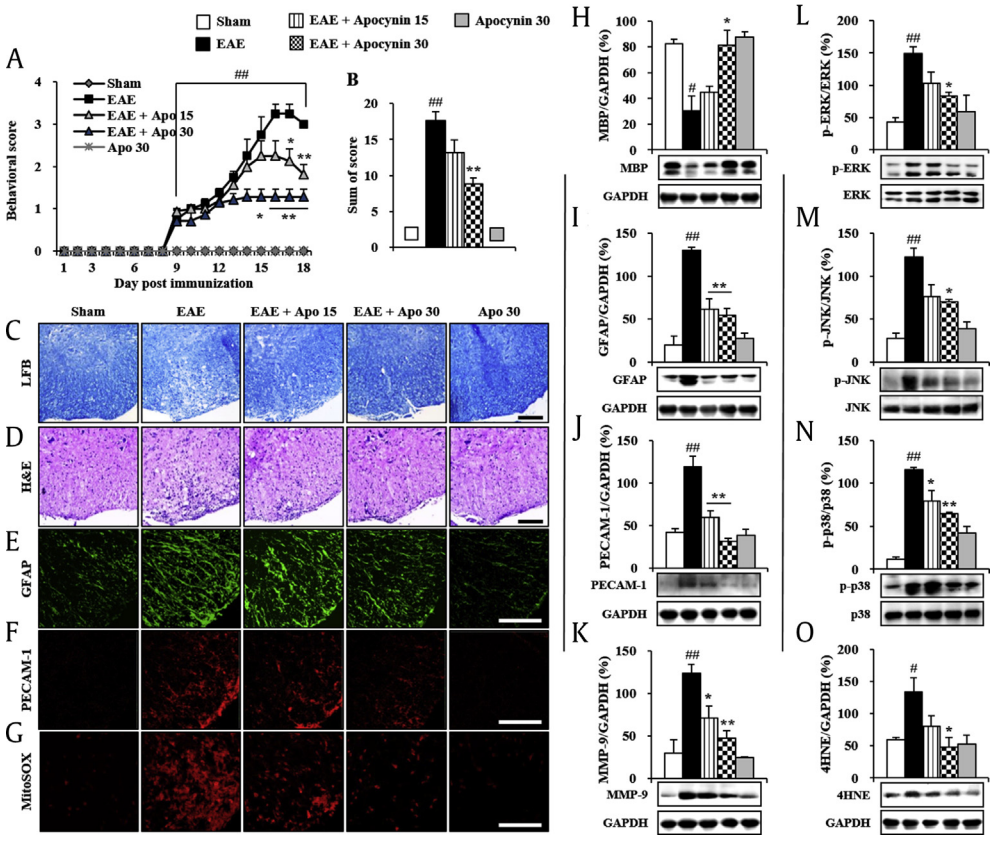

Fig. 6. NOX inhibitor mimics the beneficial effects of Rg3-KRGE in chronic EAE mice. (A and B) After EAE induction, neurological impairment was accessed once daily (n = 5 per group). Mean daily behavioral score of apocynin-administrated mice at different doses (15 and 30mg/kg) for 17 days following EAE induction (A). Scale bar = 100μm. Sum of behavioral score was assessed from day 7 until day 17 following EAE induction (B). **(C-G)** At 17 days after EAE induction, spinal cord sections (n = 3 per spinal cord) were obtained from all groups (n = 3 per group), stained with LFB (C) and H&E dye (D), analyzed by immunofluorescence staining with GFAP (E) and PECAM-1 (F) antibodies, and analyzed by MitoSOX assay (G). Scale bar = 100μm. **(H–O)** At 17 days after EAE induction, lumbar spinal cords from each group (n = 3 per group) were analyzed by Western blot to investigate protein expression levels of MBP (H), GFAP (I), PECAM-1 (J), MMP-9 (K), p-ERK (L), p-JNK (M), p-p38 (N), and 4-HNE (O) and quantified. Protein level of each target was normalized to that of GAPDH protein. Experiments were repeated three times. Data are presented as means ± SE. ANOVA test; #P < 0.05 and ##P < 0.01 vs. Sham group; *P < 0.05 and **P < 0.01 vs. EAE group.

inflammatory response, and oxidative stress in chronic EAE mice. Our results demonstrated that positive effects of Rg3-KRGE on EAE were due to improved BBB integrity via its anti-inflammation and antioxidant activities (Figs. 1–6).

Since Evan's blue dye combined with serum albumin does not cross the BBB under normal physiologic conditions, assessment of extravasated Evan's blue dye is widely used to determine permeability and BBB disruption [50]. We thus measured the extravasation of Evan's blue dye into spinal cords as an indicator of BBB permeability. As a result, Evan's blue extravasation was significantly increased in spinal cords of EAE mice compared with that of Sham control mice. In addition, levels of tight junctional molecules zonula occludens-1, claudin-3, and claudin-5 were reduced in EAE mice. However, extravasation of Evan's blue dye in spinal cords of EAE mice after administration of Rg3-KRGE was markedly reduced compared to that in EAE mice (Fig. 2). Taken together, our findings suggest that Rg3-KRGE may inhibit BBB disruption in spinal cords of chronic EAE mice via upregulation of tight junctional molecules.

In addition to BBB disruption, vascular adhesion molecules (such as PECAM-1, ICAM-1 and VCAM-1), extracellular matrix proteins (such as fibronectin), and MMP family proteins (such as MMP-9) are important components or mediators of the BBB, playing vital roles in the pathogenesis of MS and EAE [51]. MMP-9, an inducible gelatinase, is produced by CNS endothelial cells and astrocytes. Its upregulation may be a key event emerging as a central mediator of leukocyte traffic into inflamed tissues by breakdown of the BBB [52]. Targeted inhibition of MMP-9 can shelter the extracellular portion of PECAM-1 from proteolytic processing and disrupt leukocyte migration across this junctional molecule [43]. Fibronectin is secreted by human endothelial cells and astrocytes. It is important for the assembly of the basement membrane and BBB function [53–56]. In the present study, upregulated PECAM-1, fibronectin, and MMP-9 in spinal cords of chronic EAE mice were alleviated by Rg3-KRGE administration. Furthermore, apocynin prevented the enhancement in expression levels of BBB-associated proteins GFAP, PECAM-1, and MMP-9 in spinal cords of chronic EAE mice. This finding suggests that Rg3-KRGE may prevent BBB disruption in spinal cords of chronic EAE mice via regulation of vascular adhesion molecules (PECAM-1), extracellular matrix proteins (fibronectin), and MMP family.

In early stages of MS, proinflammatory enzymes (iNOS and COX-2) and cytokines (IL-1β, IL-6, and TNF-α) are released from various immune cells such as T cells and macrophages. These cytokines may cause additional damage to the CNS. However, in the later stage, anti-inflammatory enzymes (arginase-1) and cytokines (IL-4, IL-10, and IL-13) are secreted by immune cells and cytokines may be associated with resolving inflammation and tissue repair in the CNS [57]. Additionally, pro- and ant-inflammatory cytokines may be involved in maintenance of BBB integrity [58]. Thus, regulating the expression of pro- and ant-inflammatory cytokines is critical in

developing therapeutics for neurological disorders [59]. In the present study, we demonstrated that Rg3-KRGE could reduce mRNA expression of proinflammatory cytokines and enhance mRNA expression of anti-inflammatory cytokines. According to previous reports about MS and EAE, possible mechanisms underlying the anti-inflammatory and anti-oxidant effect of Rg3-KRGE might include modulating mitogen-activated protein kinases, nuclear factor (NF)-κB, and Janus kinase/signal transducers and activators of transcription signaling pathways [20,26,27,30,60]. Here, we demonstrated that administration of Rg3-KRGE could prevent upregulation in protein expression of p-ERK, p-JNK, and p-p38 in spinal cords of chronic EAE mice but apocynin inhibited them. Our results indicate that the anti-inflammatory activity of Rg3-KRGE may block BBB disruption.

Since various types of immune cells including microglia and macrophages can release pre- and anti-inflammatory mediators [57] and Rg3-KRGE is an extract containing various components, it may be difficult to identify the specific target cell type and the active component of Rg3-KRGE. Selective pharmacological inhibition on function of specific cell type might help us identify target(s) of Rg3-KRGE. *In vivo* and *in vitro* studies using treatment with potential active components are also critical in the identification of active component of Rg3-KRGE. However, since the purpose of the present study was to demonstrate the positive activity of Rg3-KRGE on BBB integrity, we did not try to explore the possible mechanisms associated with in the effect of Rg3-KRGE in the chronic EAE model. However, studies to disentangle cellular and molecular targets and the underlying mechanisms are currently on the way to provide better understanding of the preventive and therapeutic activities of Rg3-KRGE on MS and EAE.

ROS are involved in the pathogenesis of MS and EAE [47,48]. Activated NADPH oxidases (NOX) can produce ROS that are released into the intra- or extracellular space, contributing to progressive demyelination [47,48]. In the present study, we examined changes in expression levels of NOX genes in spinal cords of Rg3-KRGE-administrated EAE mice. Upregulated mRNA expressions of NOX2 and NOX4 and NADPH activity were generated in spinal cords of all EAE mice, suggesting higher oxidative stress in spinal cords of EAE mice. Interestingly, Rg3-KRGE-administration produced suppressive effects on mRNA expressions of NOX2 and NOX4 and NADPH activity, indicating that the antioxidant effect of Rg3-KRGE might exert neuroprotective effect in chronic EAE mice. However the positive effects of Rg3-KRGE was imitated by apocynin. The results suggest that Rg3-KRGE exerts beneficial effect in the chronic EAE by modulating expression of NADPH oxidase 2 and 4.

5. Conclusions

In conclusion, Rg3-KRGE may exert beneficial effects in chronic EAE therapy via its anti-inflammatory and antioxidant effects by improving BBB integrity via t through modulation of NOX2/4 expression.

Authors' contributions

MJL and JHC performed the immunohistochemistry, Western blots, and real-time PCR analysis and prepared the figures. JO and YHL performed the behavioral experiment. JGI, BJC, and SYN commented about the project. IHC conceived all experiments, analyzed the results, and wrote the manuscript. All authors have read and approved the final manuscript.

Conflicts of interest

All authors of this manuscript have no conflict of interest in this subject.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2020.09.001.

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