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Research Article

The effects of Korean Red Ginseng-derived components on oligodendrocyte lineage cells: Distinct facilitatory roles of the non-saponin and saponin fractions, and Rb1, in proliferation, differentiation and myelination

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ABSTRACT

Background: Abnormalities of myelin, which increases the efficiency of action potential conduction, are found in neurological disorders. Korean Red Ginseng (KRG) demonstrates therapeutic efficacy against some of these conditions, however effects on oligodendrocyte (OL)s are not well known. Here, we examined the effects of KRG-derived components on development and protection of OL-lineage cells.
Methods: Primary OL precursor cell (OPC) cultures were prepared from neonatal mouse cortex. The protective efficacies of the KRG components were examined against inhibitors of mitochondrial respiratory chain activity. For in vivo function of Rb1 on myelination, after 10 days of oral gavage into adult male mice, forebrains were collected. OPC proliferation were assessed by BrdU incorporation, and differentiation and myelination were examined by qPCR, western blot and immunocytochemistry.
Results: The non-saponin promoted OPC proliferation, while the saponin promoted differentiation. Both processes were mediated by AKT and extracellular regulated kinase (ERK) signaling. KRG extract, the saponin and non-saponin protected OPCs against oxidative stress, and both KRG extract and the saponin significantly increased the expression of the antioxidant enzyme. Among 11 major ginsenosides tested, Rb1 significantly increased OL membrane size in vitro. Moreover, Rb1 significantly increased myelin formation in adult mouse brain.
Conclusion: All KRG components prevented OPC deaths under oxidative stress. While non-saponin promoted proliferation, saponin fraction increased differentiation and OL membrane size. Furthermore, among all the tested ginsenosides, Rb1 showed the biggest increase in the membrane size and significantly enhanced myelination in vivo. These results imply therapeutic potentials of KRG and Rb1 for myelin-related disorders.

1. Introduction

Oligodendrocytes (OLs) are the myelin-producing cells of the central nervous system (CNS), and thus maintenance of a stable differentiated OL population is critical for efficient axonal transmission of action potentials. To produce myelin sheathes, OL precursors must proliferate, migrate to axonal tracts, and differentiate. Further, OLs must produce sufficient myelin to sustain efficient saltatory conduction of action potentials and maintain fiber tract integrity [1]. In humans, myelination begins after birth and continues throughout adulthood [2,3], and disruption of this process is associated with numerous major CNS disorders. Diseases in which progressive demyelination is considered the cardinal pathomechanism include multiple sclerosis (MS) [4,5]. Other diseases in which demyelination contributes to pathogenesis include stroke [6], spinal cord injury [7], schizophrenia [8], manic depressive

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disorder [9], Alzheimer's disease (AD) [10–13], and Parkinson's disease (PD) [14]. For instance, destruction of myelin microstructure and macrostructure is observed during the progression of AD [15] due to loss of myelin formation, maintenance, and repair [10,12,13]. Similarly, PD patients with motor symptoms but without cognitive impairment or grey matter atrophy present broad changes in white matter microstructure [16,17], suggesting that white matter dysfunction may precede neuronal loss in related grey matter regions [14]. Therefore, prevention of demyelination may be a broadly effective therapeutic strategy.

Basic mechanisms contributing to OL damage in CNS disorders include hypoxia [18], inflammation [19], glutamatergic excitotoxicity [12], and oxidative stress [19–21]. OL-lineage cells are especially susceptible to these stressors due to the high metabolic requirements for myelin production and maintenance. White matter damage in neurological disorders is often followed by OL death and insufficient regeneration from OL precursor cells (OPCs) due to oxidative stress, which interferes with OPC activation, proliferation, migration, interaction with axons, differentiation, and initiation of myelin synthesis. Successful myelin regeneration recovers axonal conduction which was lost after demyelination [22], protects axons [23,24], and neuronal cells [25].

Panax ginseng and its ginsenosides have shown their beneficial effects on neurodegenerative diseases. In PD model, *panax ginseng* extract and its components such as Rg1, Re, Rd exhibited beneficial effects such as preventing neurons from cytotoxicity, reduced locomotor dysfunction, and increased dopamine contents in the striatum [26]. *Panax ginseng* and its ginsenosides such as Rb1, Rg1, Rg2, Rg3, Rh2, and Re presented the alleviation of cognitive or pathophysiological symptoms in AD animal models as well as AD patients [26]. In relation to (de/re) myelination, Korean Red Ginseng (KRG, *Panax ginseng*) mitigates spinal demyelination in an acute experimental autoimmune encephalomyelitis (an animal model of MS) by modulating immune cells [27] and microglia/macrophages, astrocytes through p38 mitogen-activated protein kinase and nuclear factor-kB signaling pathways [28]. Moreover, gintonin, a non-saponin component of KRG, has been reported to ameliorate lead-induced impairment of myelinated fibers of Purkinje cells [29]. The effects of *Panax ginseng* on the CNS have been studied, but most investigations have focused on its neuronal functions [30], while little is known of the effects on OL lineage cells and myelin. We previously reported that gintonin, a non-saponin component of ginseng, increased OPC proliferation and late-stage OL development while reducing endoplasmic reticulum stress-induced cell death [31]. In the current study, we investigated the potential effects of KRG total extract, the saponin and non-saponin fractions as well as the ginsenosides on OPC proliferation, differentiation, pathological resistance, and myelin membrane formation.

2. Materials and methods

2.1. Mouse

For primary glia cultures, CrljOri:CD1 (ICR) mouse pups were used. For in vivo oral gavage, C57BL/6N male mice were used. Mice were purchased from ORIENT BIO Inc (Seongnam, Korea). All mice were housed under the controlled environment (23 ± 2 °C, 12-h light/dark cycle) supplied with food and water *ad libitum*. All experiments were performed in compliance with the relevant laws and institutional guidelines, and were approved by the University of Brain Education's Animal Care and Use Committee.

2.2. Reagents

KRG total extract, saponin and non-saponin fractions were provided by Korean Society of Ginseng. According to the analysis results provided by Korean Society of Ginseng, KRG contained 9.322 mg of total amount for Rg1, Rb1, and Rg3 per gram. Non-saponin fraction included 12.98% crude protein, 71.39% carbohydrate, 338.93kcal/100g calorie, 1.54 % glucose, 2.84% fructose, 1.62% sucrose, 3.89% maltose, 8.74% moisture, 0.16% crude fat, 6.73% crude ash, 154 mg/kg sodium. Saponin fraction contained 6.78 mg/g Rb1, 2.57 mg/g Rc, 2.35 mg/g Rb2, 1.83 mg/g Re, 1.63 mg/g Rg3s, 1.47 mg/g Rg1, 1.13 mg/g Rf, 0.95 mg/g Rg2s, 0.9 mg/g Rg3r, 0.73 mg/g Rd, and 0.45 mg/g Rh1. Rb1 and Rg3 were purchased from Sigma-Aldrich (St. Louis, US), while other ginsenosides were purchased from ChemFaces (Wuhan, China). U0126 and LY294002 were purchased from Cell Signaling Technology (CST, Danvers, US). Rotenone, antimycin, and oligomycin were purchased from Sigma-Aldrich. Rabbit anti-ERK-1/2 and rat anti-myelin basic protein (MBP) polyclonal antibodies were purchased from Sigma-Aldrich. Rabbit anti-phospho-ERK1/2, anti-AKT, and anti-phospho-AKT antibodies were purchased from CST. Buffers and reagents for primary cell culture were purchased from Gibco, Sigma-Aldrich, or Protein Tech.

2.3. Preparation of primary mixed glial cultures and OPC isolation

Primary glial mixed cultures and OPC isolation were prepared as described previously [31,32]. Briefly, the cerebral cortex was isolated from brains of postnatal day 0–1 ICR mouse pups, dissociated for glial mixed culture, and maintained in proliferation medium (DMEM/F-12 medium containing 10% fetal bovine serum, 5% horse serum and 1xpenicillin-streptomycin). After ten days, OPCs were isolated by vigorous manual shaking and subjected for experiments. For proliferation, the cells were maintained in the proliferation medium. For differentiation, the cells were maintained in differentiation medium (DMEM containing 1 × B-27 supplement, 1 × Glutamax, 1 × penicillin–streptomycin, 1% horse serum, 1 × sodium pyruvate, 0.34 μg/ml T3, and 0.4 μg/ml T4). Medium was changed into fresh medium every other day.

2.4. In vivo mouse experiment

Seven week C57BL/6N male mice were placed 3-4 mice per cage with environmental enrichment including TAPVEI cube, TAPVEI labyrinth, Safe Harbor Mouse Retreat (WOOJUNGBIO, Korea) and subjected to oral gavage of the indicated concentration of Rb1 or distilled water every morning for 10 consecutive days. On the next day of the final oral gavage, mouse brains were isolated and forebrains were subjected to analyze by quantitative real time PCR (qPCR) and western blot (WB) (Fig. 5A).

2.5. Cytotoxicity test

For cytotoxicity test, lactate dehydrogenase (LDH) release was measured with a commercial detection kit (Takara Bio).

2.6. Proliferation assay

For proliferation assay, BrdU incorporation assays were conducted according to the manufacturer's instructions (Millipore, Darmstadt, Germany).

2.7. Quantitative real-time PCR

Total RNA was extracted from OPC cultures or brain tissues using TRI reagent (Sigma-Aldrich) and reverse transcribed into cDNA using the Superscript First-Strand Synthesis System (Thermo Fisher). qPCR was conducted using PowerUp SYBR Green Master Mix (Life Technologies) and the primer sequences are listed in Supplementary table 1.

2.8. Western blot

Cells treated as indicated were collected directly in sample buffer. Brain tissues were lysed in Ripa buffer (ATTO, WSE-7420 EzRIPA Lysis kit) and centrifuged, then the supernant was diluted with 5xSample buffer, boiled at 98 °C for 3 min. Total proteins were separated by sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) on 12.5% gel for MBP and 10% gel for other proteins. Separated proteins were transferred onto PVDF membranes, which were then blocked by Tris-buffered saline containing Triton-X (TBST)/5% bovine serum albumin for 1 h at room temperature (RT). Membranes were incubated with the primary antibody at 4 °C overnight, washed and incubated in secondary antibody, and rewashed in TBST. Immunolabeled proteins were visualized by enhanced chemiluminescence reagent and captured using Amersham Imager 600 (GE Healthcare). Images were analyzed by using ImageJ.

2.9. Immunofluorescence and image analysis

Treated cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde at RT, washed, blocked for 45 min at RT in PBS/5% donkey serum/0.5% Triton X-100, then incubated overnight at 4 °C with the primary antibody diluted in blocking buffer. Immunolabeled cells were washed with PBS, incubated with secondary antibody for 45 min at RT, washed again in PBS, and mounted in antifade mounting medium. Pictures were captured using an LSM700 confocal microscope (Carl Zeiss, Germany). Membrane area was measured from images using ImageJ.

2.10. Statistical analyses

Statistical analyses were performed using one or two-way analysis of variance (ANOVA) with Holm-Sidak *post hoc* tests by using Sigmaplot. *P* values less than 0.05 were considered as statistically significant.

3. Results

3.1. The non-saponin fraction increases OPC proliferation

To investigate the effects of the KRG-derived components on OPC proliferation, we conducted BrdU assays following treatment at DIV1 with 0, 1, 10, or 100 μg/ml total extract, saponin, or non-saponin fraction for 24 h (Fig. 1A, Supplementary Fig. 1). The non-saponin fraction but not the total extract or saponin fraction increased BrdU incorporation, indicating enhanced proliferation rate.

3.2. The saponin fraction increases OL differentiation and its membrane formation

To assess the effects of KRG-derived components on OL differentiation, we measured the expression levels of MBP mRNA following 4 days of culture in differentiation medium containing 0, 1, 10, 20, or 100 μg/ml KRG total extract, saponin, or non-saponin fraction. The saponin fraction significantly increased MBP expression at all tested concentrations compared to control cultures (Fig. 1B), while there were no significant changes in MBP mRNA expression following culture in total KRG extract or non-saponin fraction. Further, the saponin fraction also increased MBP protein expression in differentiated OLs (DIV7), although the change did not reach statistical significance (Fig. 1C–D). To analyze the effects of the saponin fraction on OL membrane formation, OPCs were cultured in differentiation medium with saponin fraction (0, 1, 10 μg/ml) from DIV1 until DIV7 (with medium and drug exchange every other day), and fixed for immunofluorescence staining of MBP (Fig. 1E). Compared to control DIV7 cells, those treated with saponin fraction showed a significant increase in MBP-immunopositive area (Fig. 1F).

3.3. Contributions of ERK and AKT signaling pathways to modulation of OPC proliferation and differentiation by KRG-derived components

Both ERK [33–36] and AKT [37] signaling pathways are critical regulators of OL development and they crosstalk each other [38]. Results presented thus far indicate that the non-saponin fraction promotes OPC proliferation while the saponin fraction promotes differentiation. To examine if these effects are mediated by ERK and/or AKT signaling, we first measured changes in pathway activities during treatment. Treatment of OPCs at DIV1 with 1 μg/ml non-saponin fraction transiently enhanced AKT phosphorylation, with a significant increase noted as early as 10 min post-application before returning to baseline within 6 h (Fig. 2A and B). ERK phosphorylation was also enhanced, without significant differences (Fig. 2C). The summed values showed a significant increase at 10 min post-application (Fig. 2D). Treatment of differentiated DIV3 OLs with the saponin fraction significantly increased the summed phosphorylation values at 10 min post-application before returning to baseline within 1h at the concentration of 100 μg/ml (Fig. 2E–H). Finally, KRG total extract (10 μg/ml, Fig. 2I-L) also slightly increased AKT and ERK phosphorylation, but none of the changes reached significant differences (Fig. 2I-L).

To further distinguish the contributions of AKT and ERK signaling pathways to enhanced OPC proliferation by the non-saponin fraction (Fig. 1A), we pretreated DIV1 OPCs with either the ERK1/2 inhibitor U0126 for 2 h, the PI3K/AKT inhibitor LY294002 for 1 h, or vehicle prior to non-saponin fraction treatment (1 μg/ml) in the continued presence or absence of the pretreatment agent for an additional 24 h. Low concentrations and brief treatment with either inhibitor was sufficient to significantly reduce the non-saponin fraction-mediated increase in OPC proliferation as measured by BrdU assay (Fig. 2M). Similarly, to distinguish the contributions of AKT and ERK signaling pathways to saponin fraction-induced OL differentiation (Fig. 1), we pretreated differentiated OLs (DIV3) with 1 μM U0126 for 2 h or 10 μM LY294002 for 1 h prior to incubation in 10 μg/ml saponin fraction or 10 μg/ml KRG total extract for 24 h, and measured MBP expression by qPCR at DIV4. While the total extract did not reach the statistical significance, saponin fraction treatment significantly increased MBP mRNA expression, consistent with our previous experiments (Fig. 1), and both inhibitors significantly reduced MBP mRNA expression (Fig. 2N). Collectively, these results suggest that both AKT and ERK signaling pathways mediate enhanced OPC proliferation by the non-saponin fraction and enhanced OL differentiation by the saponin fraction.

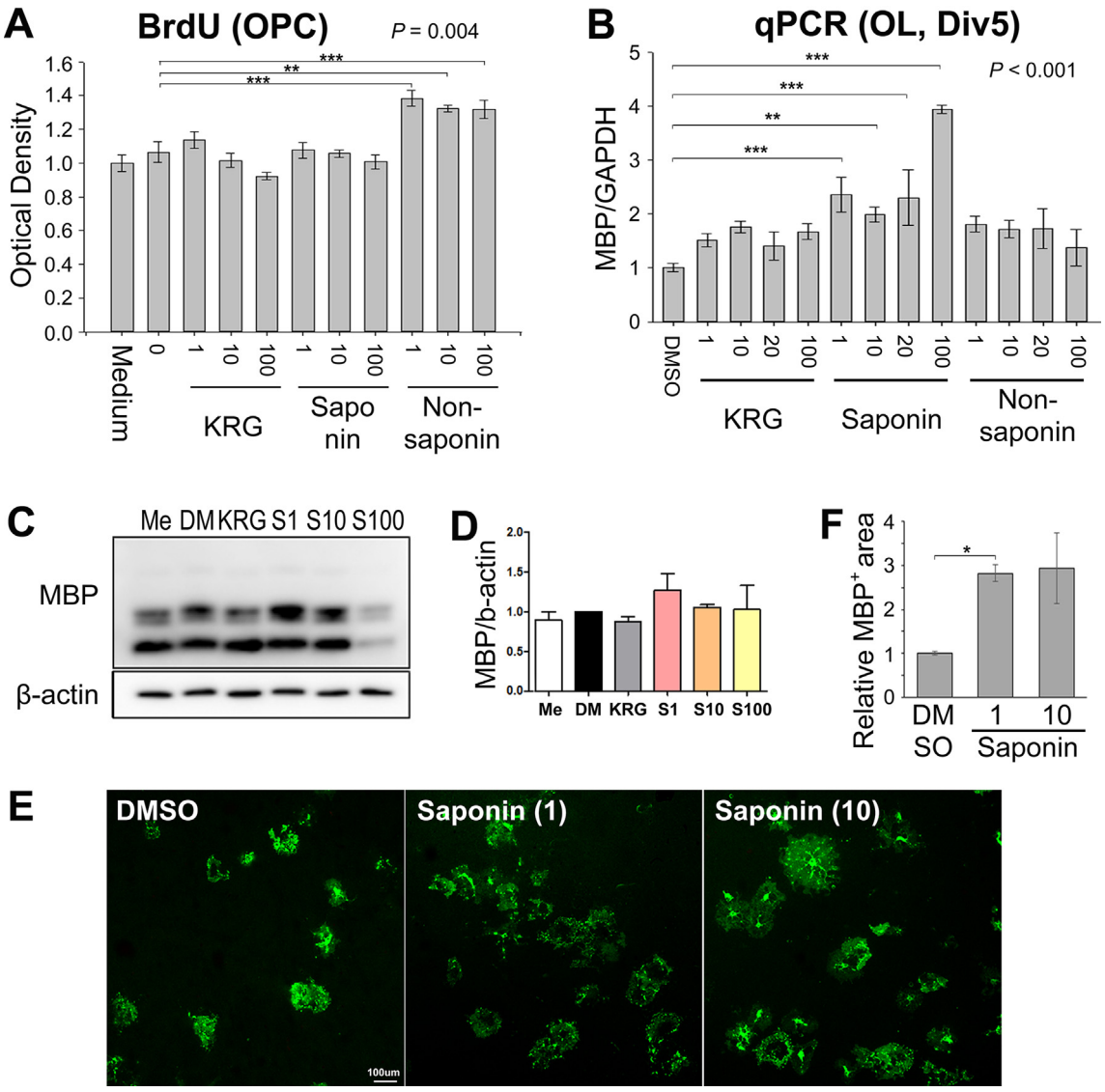

Fig. 1. Promotion of OPC proliferation and differentiation by the non-saponin and the saponin fraction, respectively. (A) Effects of KRG-derived components on OPC proliferation as assessed by BrdU incorporation assay. Cells were treated with the indicated preparation for 24 h at DIV1 and the BrdU assay was performed at DIV2. Two-way ANOVA results: $^{**}P = 0.004$. Post-hoc test using the Holm-Sidak method: 0 (vehicle) vs. 1 μg/ml, $^{***}P < 0.001$; 0 vs. 100 μg/ml, $^{***}P < 0.001$; 0 vs. 10 μg/ml, $^{**}P = 0.001$). (B) Effects of KRG-derived components on OPC differentiation as measured by qPCR of MBP mRNA expression at DIV5. Cells were treated with the indicated doses (μg/ml) in differentiation medium from DIV1 to DIV5. Results of two-way ANOVA: $^{***}P < 0.001$. Post-hoc analysis using the Holm-Sidak method: 1 μg/ml saponin vs. DMSO (vehicle), $^{***}P < 0.001$; 10 μg/ml vs. DMSO, $^{**}P = 0.006$; 20 μg/ml vs. DMSO, $^{***}P < 0.001$; 100 μg/ml vs. DMSO, $^{***}P < 0.001$. (C-D) Effects of KRG-derived components on MBP protein expression as assessed by Western blotting. Cultures were treated in differentiation medium from DIV1 to DIV7. (E-F) Changes in oligodendrocyte (OL) membrane size. Cells were treated with the saponin fraction in differentiation medium from DIV1 to DIV7. (E) Representative low-magnification images (× 10 × 0.5) of cultures treated with DMSO (left), 1 μg/ml (middle), or 10 μg/ml (right) saponin fraction. Scale bar, 100 μm. (F) Relative size of MBP-positive area per image field. Results of one-way ANOVA: $^{*}P = 0.049$. Post-hoc analysis using the Holm-Sidak method: 1 μg/ml saponin fraction vs. DMSO, $^{*}P = 0.036$. All graphs are presented as mean ± standard error of the mean of N = 3 independent primary cultures per group. OPC, oligodendrocyte precursor cell; DIV, days in vitro; ANOVA, analysis of variance; KRG, Korean Red Ginseng; qPCR, quantitative real time PCR; MBP, myelin basic protein; Me, Medium; DM, DMSO; S, Saponin.

3.4. KRG-derived components prevent OPC death under chemical hypoxia

Mitochondrial dysfunction and ensuing oxidative stress in a central pathogenic process are responsible for death of OL-lineage cells in neurological diseases involving inflammation, demyelination, and axonal injury [39,40]. To examine if KRG-derived components can protect against oxidative stress and OL-lineage cell death, we measured LDH release from OPCs and OLs in response to mitochondrial respiratory chain inhibitors (inducing chemical hypoxia) following pretreatment with vehicle (control), KRG total extract, saponin fraction, or non-saponin fraction. Chemical hypoxia was induced by the mitochondrial electron transport chain complex I inhibitor rotenone, the complex III inhibitor antimycin, or the ATP synthase inhibitor oligomycin [41]. Appropriate test concentrations were first determined by administering multiple inhibitor doses to OPCs at DIV1 and OLs at DIV5 for 24h, followed by LDH assay (Fig. 3A–F). In OPC cultures, rotenone induced 24% cell death at 5 μM, 30% at 10 μM, 49% at 20 μM, and 73% at 40 μM (Fig. 3A), while corresponding death rates in OL cultures were 39%, 40%, 52%, and 53% (Fig. 3D). In OPC cultures, antimycin induced 26% cell death at 25 μM, 29% at 50 μM, 58% at 100 μM, and 102% at 200 μM (Fig. 3B), while corresponding rates for OLs were 37%, 51%, 51%, and 99% (Fig. 3E). Finally, oligomycin induced 20% OPC death at 2.5

μM, 67% at 5 μM, 73% at 10 μM, and 84% at 20 μM (Fig. 3C), while corresponding values for OLs were 15%, 29%, 71%, and 105% (Fig. 3F). Based on these results, 20 μM rotenone, 100 μM antimycin, and 10 μM oligomycin were chosen as test doses for both OPC and OLs. Cultures of OPCs were pretreated with KRG-derived components for 24 h at DIV1 and then with inhibitors for additional 24 h, followed by LDH assays (Fig. 3G–I). Rotenone-induced cell death was significantly reduced by 47% (total extract), 39% (saponin), and 28% (non-saponin) (Fig. 3G). Similarly, antimycin-induced toxicity was reduced by 63% (total extract), 48% (saponin), and 39% (non-saponin) (Fig. 3H). Similarly, oligomycin-induced toxicity was reduced by 43% (total extract), 38% (saponin), and 44% (non-saponin) (Fig. 3I). In differentiated OLs, KRG-derived components were administered for 24 h at DIV4 and inhibitors for an additional 24 h at DIV5, followed by LDH assays at DIV6 (Fig. 3J-L). In contrast to OPCs, the components did not induce significant protective efficacy under chemical hypoxia in mature OLs (Fig. 3J-L). To reveal potential responses that may account for the OPC-protective functions of KRG-derived components, we examined changes in antioxidant gene expression. The results showed that KRG total extract and saponin fraction significantly increased expression of the antioxidant NQO1 in OPCs (Fig. 3M). In contrast, expression of the antioxidant GCLC was not influenced by any of these treatments (Fig. 3N).

3.5. Rb1 enhances OL membrane formation in primary OL cultures

Finally, we examined the effects of individual ginsenosides on OL membrane formation (Fig. 4). According to the analytic information provided by Korean Ginseng Society, the ginsenoside constituents of the saponin fraction are as follows: Rb1, Rc, Rb2, Re, Rg3s, Rg1, Rf, Rg2s, Rg3r, Rd, and Rh1 (refer to Materials and Methods section). OL cultures were treated with each ginsenoside from DIV1 to DIV7, and OL membrane formation was assessed by MBP-positive membrane size (Fig. 4). The test concentrations were determined by comparison among 2 or 3 serial dilutions (1, 10, 100 μM) (data not shown). Compared to DMSO control, the saponin fraction (1 μg/ml) and Rb1 (1 μg/ml) increased MBP-immunopositive area by 2.9-fold and 2.6-fold, respectively, while the other ginsenosides, total KRG extract, and non-saponin fraction had no significant effects (Fig. 4Z). In qPCR investigating gene expression pattern related with pro-myelin production on differentiated OLs, Rb1 increased the expression of Myrf, a transcription factor for pro-myelin production, by 1.3-fold, without a significant difference at DIV3 (Supplementary Fig. 2).

3.6. Rb1 enhances myelination in adult mice

To investigate whether Rb1 also functions in vivo, we performed oral gavage experiments on adult mice. During adulthood, central nervous system myelin keeps its plasticity in response to environmental stimuli [42]. We allowed adult mice to facilitate brain plasticity by using environmental enrichment [43]. Three to four 7 week-old C57BL/6N male mice were placed together in one cage containing environmental enrichment (refer to Materials and Methods section for detail) and subjected to oral gavage of distilled water or Rb1 (0.1, 1, 5 mg Rb1/kg body weight) every morning for 10 days. On the next day of the final oral gavage, mice were sacrificed to isolate forebrains, followed by sample lysis for qPCR or western blot (Fig. 5A). The MBP expressions of total RNA as well as protein were significantly increased in 0.1 mg/kg Rb1-feeding mice compared to the DW-feeding mice (Fig. 5B–D). MBP protein expression was also enhanced in other Rb1 concentration (1, 5 mg/kg)-feeding mice compared to the control mice (Fig. 5D). The MBP expression level was reduced in high concentration of Rb1 (Fig. 5B, D), indicating potential toxic effect of Rb1 in high concentration (1, 5 mg/kg), but not in low concentration (0.1 mg/kg).

4. Discussion

The proliferation of OPCs is critical for myelin regeneration following injury [44]. Therefore, acceleration of OPC proliferation by non-saponin fractions (Fig. 1) could contribute to enhance remyelination under pathological conditions. This effect was mediated by activation of AKT and ERK signaling pathways (Fig. 2), consistent with previous reports that both cascades interact to regulate OPC proliferation [33–35,45]. These findings are also consistent with our previous findings using the gintonin in OPCs [31] as well as with reports that the non-saponin fraction increased the proliferation rate of neuroblastoma cells [46]. Gintonin is a lysophosphatidic acid (LPA)-ginseng protein complex that selectively activates LPA receptors (LPARs) [47], which are known regulators of cell proliferation [48,49]. OPCs express the highest levels of LPARs 2 and 3 among brain cell types [50], and gintonin activates these receptors with high affinity [51]. Therefore, it is reasonable to speculate that the non-saponin fraction accelerated OPC proliferation at least in part by activating both LRAR and AKT/ERK signaling cascades.

Unlike non-saponin fraction, the saponin fraction enhanced differentiation and membrane formation (Figs. 1 and 4). Remyelination failure due to limited OL differentiation has been reported in several demyelinating conditions. In MS, for example, OPCs remain in a quiescent state, resulting in remyelination failure following demyelinating injury. In addition, Aβ deposition directly induces OPC senescence in both AD patients and model mice, resulting in remyelination failure [42]. Therefore, saponin fractions may have broad therapeutic efficacy against neurological diseases by re-inducing myelin formation.

These pro-differentiation responses also appeared to be mediated by AKT and ERK signaling pathways (Fig. 2), consistent with previous studies implicating ERK1/2 and Akt/mTOR signaling individually in OL differentiation, initiation of myelination, and myelin membrane growth [36,52] as well as studies reporting mutual interactions between ERK1/2 and AKT/mTOR signaling in the regulation of OL differentiation [38]. We further show that the effect of saponin on OL differentiation and membrane formation is probably contributed by the effect of ginsenoside Rb1 (Fig. 4). Rb1 also increased the total RNA as well as protein expression of MBP in adult mouse forebrains under environmental enrichment (Fig. 5), supporting the pro-myelin function of Rb1. In experimental autoimmune encephalomyelitis (EAE) which accompanies demyelination, Rb1 involved immune regulation, thereby relieving symptoms [27]. In addition, Rb1 also reduced hippocampal CA1 neuronal death and cognitive dysfunction in rats receiving hippocampal injections of Aβ1–40. Thus, Rb1 may have multimodal beneficial effects under environmentally enriched conditions as well as demyelinating conditions by promoting myelination and rescuing both OPCs and neurons from cytotoxic stress.

In this study, all KRG-derived components exhibited cytoprotective function against oxidative stress-induced cell death in OPCs but not in mature OLs (Figs. 3 and 6). The activities of the antioxidant enzymes superoxide dismutases, glutathione peroxidase, and catalase are enhanced in mature OLs compared to OPCs [53], indicating distinct protective mechanism compared to OPCs. The precise mechanism for OPC-protective effects was further examined by measuring expression levels of antioxidants under chemical hypoxia. The expression of NQO1 but not GCLC was significantly upregulated in OPCs by treatment with KRG total extract or the saponin fraction (Fig. 3M, N), which is surprising because both genes are under control of the transcription factor Nrf2 [54,55]. We

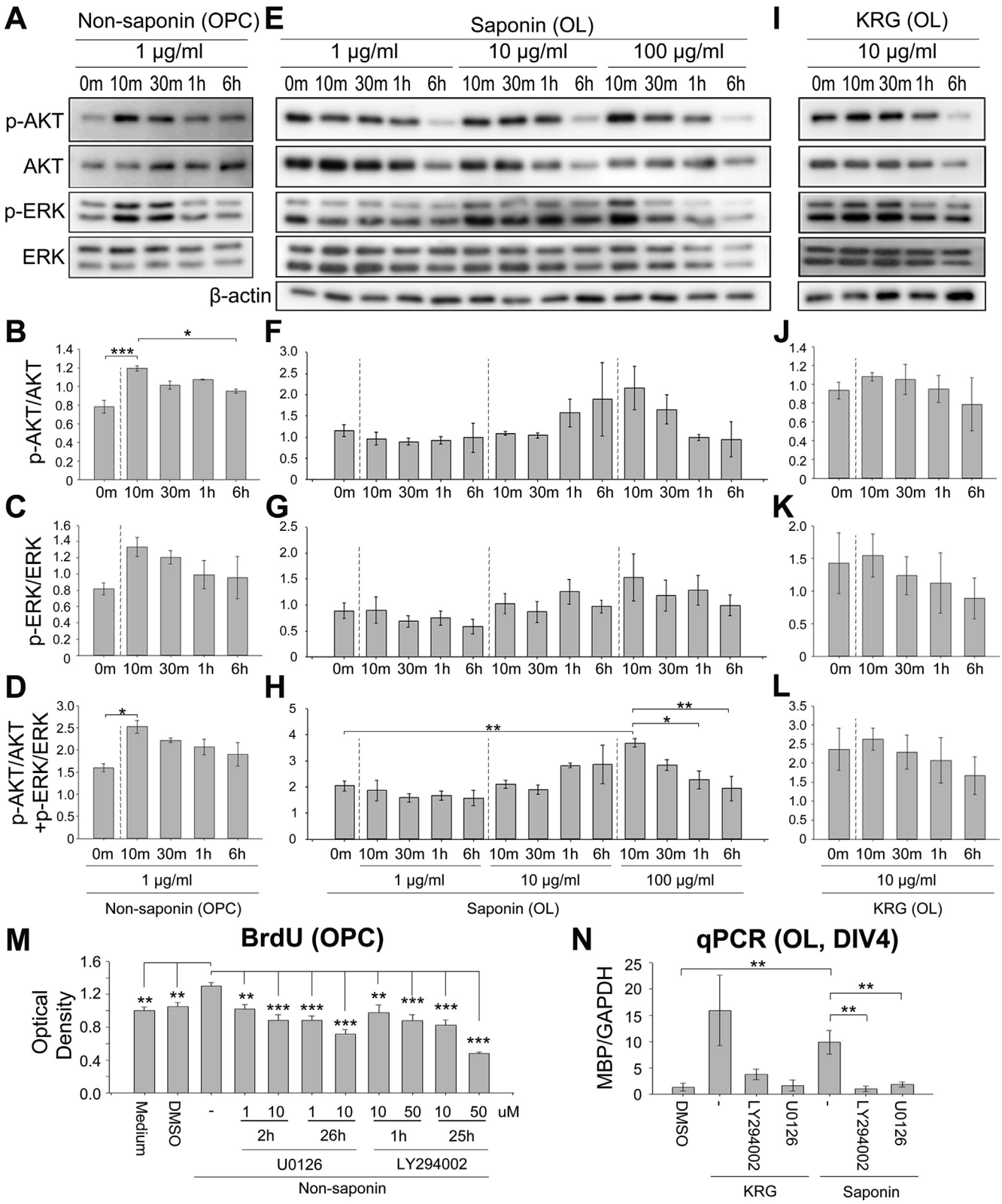

Fig. 2. Effects of KRG-derived components on AKT and ERK signaling in OL-lineage cells. Cultured cells were collected after 0 min, 10 min, 30 min, 1 h, and 6 h of treatment with the indicated components and subjected to western blotting (A, E, I) or image analysis (B-D, F–H, J-K). (A-D) OPCs were treated with non-saponin fraction in proliferation medium at DIV1. (B) Results of one-way ANOVA for AKT: $^{***}P < 0.001$. Results of pair-wise comparisons using the Holm-Sidak test: 0 vs. 10 min, $^{***}P < 0.001$; 10 min vs. 6 h, $^{*}P = 0.012$. (D) Results of one-way ANOVA for AKT + ERK: $^{*}P = 0.026$. Results of pair-wise comparisons using the Holm-Sidak test: 0 vs. 10 min, $^{*}P = 0.010$. (E-H) Differentiated OLs were treated with 1, 10, or 100 μg/ml saponin fraction in differentiation medium at DIV3. (H) Results of two-way ANOVA for AKT + ERK: $^{**}P = 0.008$. Results of pair-wise comparisons using the Holm-Sidak test: 0 vs. 10 min at 100 μg/ml, $^{**}P = 0.007$; 10 min vs. 1 h, $^{*}P = 0.025$; 10 min vs. 6 h, $^{**}P = 0.004$. (I-L) Differentiated OLs at DIV3 were treated with KRG total extract (10 μg/ml) in differentiation medium for the indicated durations. There were no significant changes in AKT or ERK phosphorylation. (M) OPCs were treated with the ERK1/2 inhibitor U0126 (U, 1 or 10 μM for 2 h) or the PI3K inhibitor LY294002 (L, 10 or 50 μM for 1 h) at DIV1, then with 1 μg/ml non-saponin fraction (NS) alone or together with the indicated

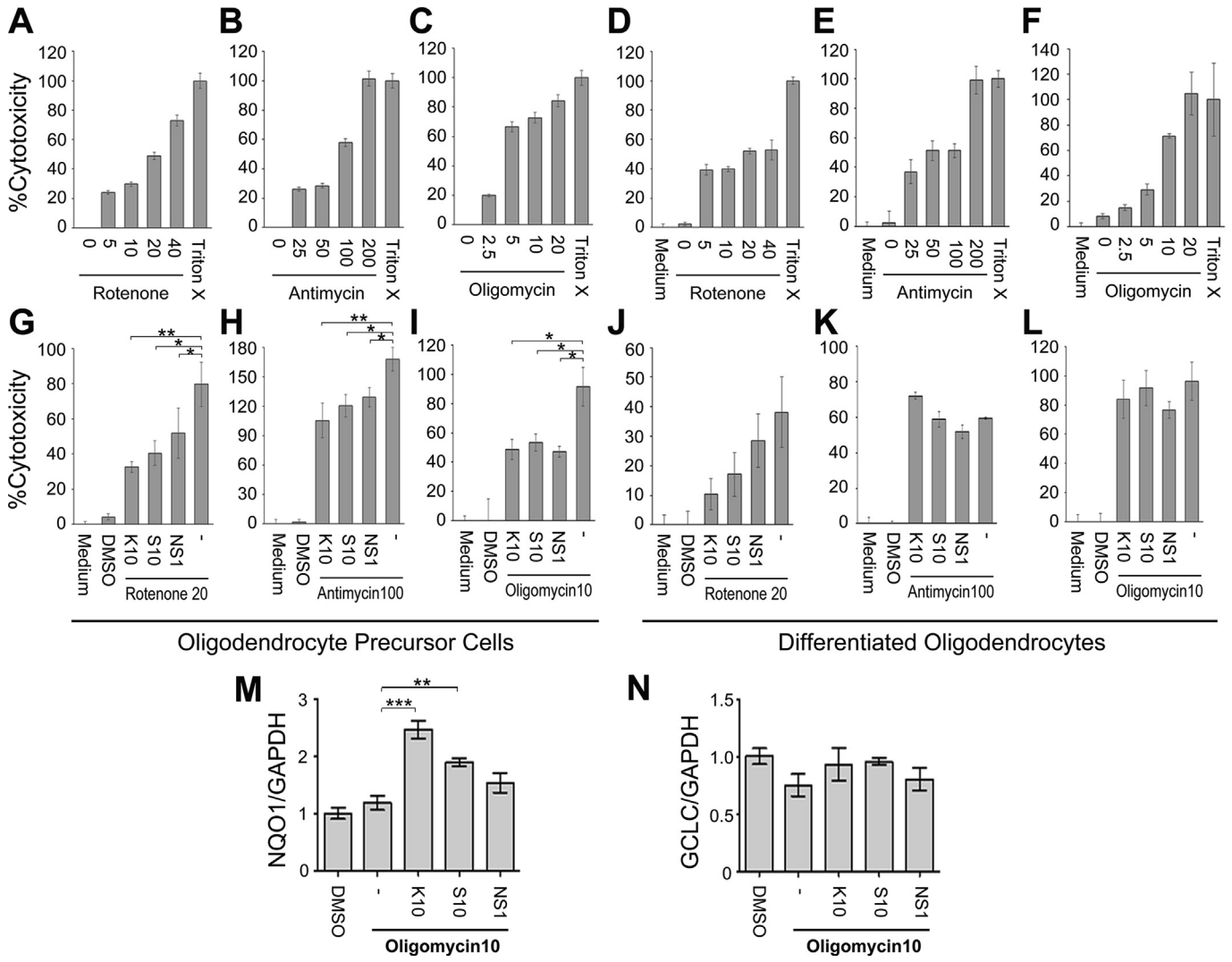

Fig. 3. Cytoprotective efficacies of KRG-derived components under chemical hypoxia. (A-F) Dose-dependent cytotoxicities of the mitochondrial complex I electron transport chain inhibitor rotenone (Rot), the mitochondrial electron transport chain complex III inhibitor antimycin (Ant), and the ATP synthase proton channel blocker oligomycin (Oli). Results are presented as % cell death vs. dose in μM. (A-C) OPCs were treated with inhibitors at DIV1 for 24 h, then subjected to LDH assay at DIV2. (D-F) Differentiated OLs were treated with inhibitors at DIV5 for 24 h, then subjected to LDH assays at DIV6. (G-L) Protective efficacies of KRG-derived components against cell death induced by inhibitors. (G-I) OPCs were treated with KRG-derived components at DIV1 for 24 h, then incubated with inhibitors at DIV 2 for an additional 24 h, followed by LDH assay at DIV3. (G) Results of one-way ANOVA for Rot: $^{***}P < 0.001$. Results of pair-wise comparisons using the Holm-Sidak test: Rot vs. Rot/R10, $^{**}P = 0.006$; Rot vs. Rot/S10, $^{*}P = 0.013$; Rot vs. Rot/NS1, $^{*}P = 0.037$. (H) Results of one-way ANOVA for Oli: $^{***}P < 0.001$. Results of pair-wise comparisons using the Holm-Sidak test: Oli vs. Oli/K10, $^{*}P = 0.021$; Oli vs. Oli/S10, $^{*}P = 0.020$; Oli vs. Oli/NS1, $^{*}P = 0.025$. (I) Results of one-way ANOVA for Ant: $^{***}P < 0.001$. Results of pair-wise comparisons using the Holm-Sidak test: Ant vs. Ant/K10, $^{**}P = 0.005$; Ant vs. Ant/S10, $^{*}P = 0.019$; Ant vs. Ant/NS1, $^{*}P = 0.027$. (J-L) Differentiated OLs were treated with KRG-derived components at DIV4 for 24 h, then incubated with inhibitors at DIV 5 for an additional 24 h, followed by LDH assays at DIV6. (M-N) OPCs were treated with KRG-derived components at DIV1 for 24 h, then incubated with inhibitors at DIV2 for an additional 24 h, followed by sample collection for qPCR at DIV3. (M) Relative expression of NQO1. Results of one-way ANOVA: $P < 0.001$. Results of pair-wise comparisons using the Holm-Sidak test: Oli vs. K10/Oli, $^{***}P < 0.001$; Oli vs. S10/Oli, $^{**}P = 0.009$. (N) Relative expression of GCLC. $P = 0.345$. All graphs are presented as mean ± standard error of the mean of N = 3 independent primary cultures per group. LDH, lactate dehydrogenase; K, KRG total extract; S, saponin; NS, non-saponin; OL, oligodendrocyte; DIV, days in vitro; ANOVA, analysis of variance.

inhibitor. At DIV2, BrdU assay was performed. Results of one-way ANOVA: $^{***}P < 0.001$. Results of pair-wise comparisons using the Holm-Sidak test: NS vs. Medium, $^{**}P = 0.004$; NS vs. DMSO, $^{**}P = 0.006$; NS vs. NS/U1 (2 h), $^{**}P = 0.005$; NS vs. NS/U10 (2 h), $^{***}P < 0.001$; NS vs. NS/U1 (26 h), $^{***}P < 0.001$; NS vs. NS/U10(26h), $^{***}P < 0.001$; NS vs. NS/L10(1h), $^{**}P = 0.003$; NS vs. NS/L50 (1 h), $^{***}P < 0.001$; NS vs. NS/L10 (25 h), $^{***}P < 0.001$; NS vs. NS/L50 (25 h), $^{***}P < 0.001$. (N) Cells under differentiation conditions (differentiation medium) were pretreated with inhibitor (1 μM U0126 for 2 h or 10 μM LY294002 for 1 h) at DIV1, then treated with the 10 μg/ml KRG or saponin, followed by collection at DIV4 for qPCR. Results of one-way ANOVA: $^{**}P = 0.002$. Results of pair-wise comparisons using the Holm-Sidak test: DMSO vs. S, $^{**}P = 0.002$; S vs. S/L, $^{**}P = 0.003$; S vs. S/U, $^{**}P = 0.002$. All graphs are presented as mean ± standard error of the mean of N = 3 independent primary cultures per group. KRG, Korean Red Ginseng; ERK, extracellular regulated kinase; OL, oligodendrocyte; DIV, days in vitro; ANOVA, analysis of variance; OPC, oligodendrocyte precursor cell; NS, non-saponin; S, saponin.

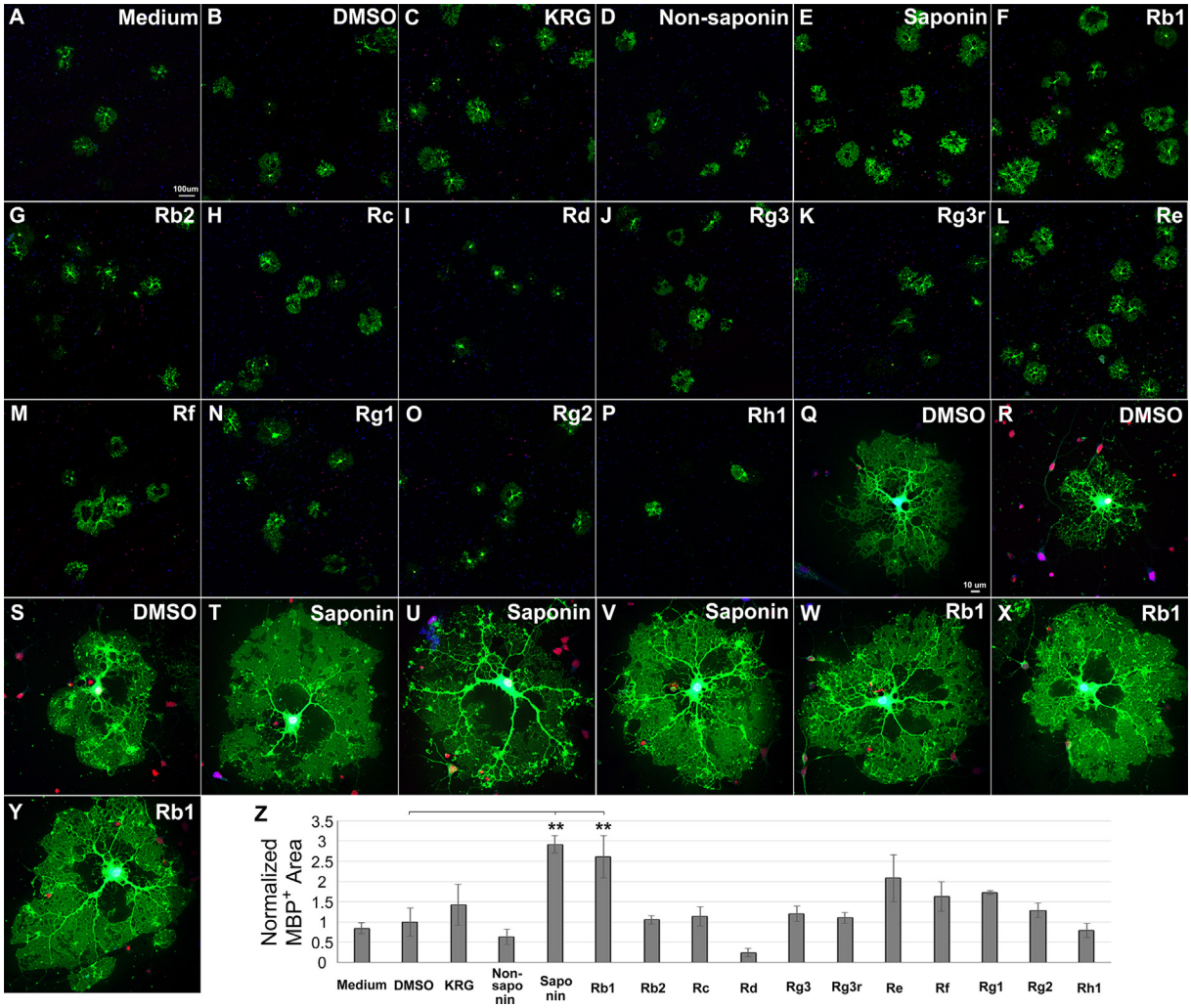

Fig. 4. Effects of individual ginsenosides on OL membrane size. OLs were treated with the indicated extract or ginsenoside under differentiation conditions from DIV1 to DIV7 (with medium change every other day). Cells were fixed at DIV7 for MBP immunocytochemistry. Final concentrations were as follows. KRG total extract, 10 μg/ml; non-saponin, 1 μg/ml; saponin, 1 μg/ml; Rb1, 1 μg/ml; Rb2, 10 μM; Rc, 10 μM; Rd, 100 μM; Rg3, 10 μM; Rg3r, 10 μM; Re, 10 μM; Rf, 100 μM; Rg1, 10 μM; Rg2, 100 μM; Rh1, 100 μM. (A-P) Representative low magnification culture images for membrane size analysis (× 10 × 0.5). Scale bar = 100 μm. (Q-Y) High resolution (× 63 × 0.5) pictures of OLs treated with DMSO (Q-S), saponin (T-V), or Rb1 (W–Y). Scale bar = 10 μm. (Z) MBP-positive area per field normalized to the control (DMSO). Results of one way ANOVA: ***$P < 0.001$. Results of pair-wise comparisons using the Holm-Sidak test: DMSO vs. saponin, **$P = 0.001$; DMSO vs. Rb1, **$P = 0.007$. The graph is presented as mean ± standard error of the mean of N = 3 independent primary cultures per group. OL, oligodendrocyte; DIV, days in vitro; MBP, myelin basic protein; ANOVA, analysis of variance.

speculate that GCLC may be induced transiently within far less than 24 h or with a delay greater than 24 h since the treatment of KRG-derived components. In either case, such changes would not be detected using our current study design. Alternatively, NQO1 may be increased selectively by mechanisms other than through Nrf2, such as activation of the Ah receptor [54]. The non-saponin fraction also induced cytoprotection without altering NQO1 and GCLC expression levels (Fig. 3M, N), suggesting a different mechanism from the saponin's mechanism.

The concentration of the non-saponin component gintonin is 0.2% (wt/wt) in total ginseng, while total ginsenoside concentration is 3%–4% (wt/wt) 57. Curiously, the effects of non-saponin and saponin fractions were distinct from those of total extract (Fig. 1) despite sharing certain components. These differences may reflect enrichment in the fractions compared to total extract or the presence of other components in total extract that inhibit bioactivity. Nonetheless, the total extract demonstrated the highest cytoprotection among the three (Fig. 3), suggesting that components lost in the saponin and/or non-saponin fractions but present in the total extract may contribute the cytoprotective effects.

Many neurological disorders involve myelin degeneration. For efficient myelin regeneration, OPC proliferation, differentiation, and/or myelin formation must be sustained. In this study, we found enhanced OPC proliferation in the presence of the non-saponin fraction, elevated OL differentiation and greater membrane size in the presence of the saponin fraction and Rb1, and increased cytoprotective capacities in the presence of all three preparations (including the KRG total extract). Finally, we found the pro-myelin function of Rb1 in adult mouse brains. These observations suggest that KRG-derived components can induce functional improvements required for OL survival and myelination, warranting further studies to validate the effects of KRG-derived components on OL-lineage cells in vivo demyelination model.

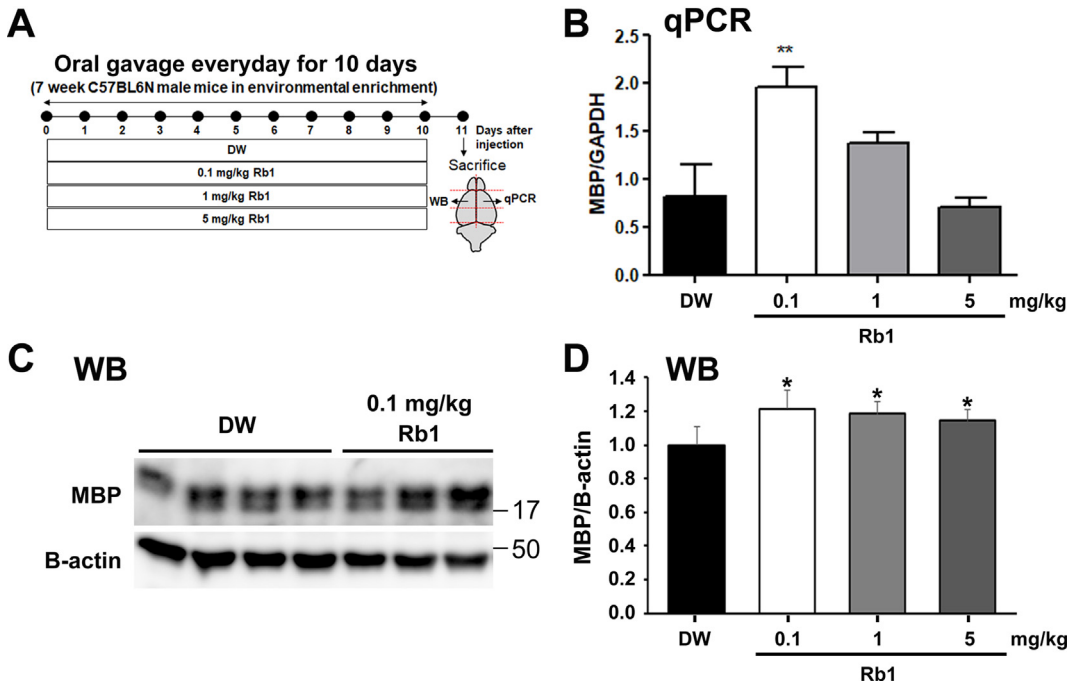

Fig. 5. Facilitation of myelin formation in adult mice by Rb1 treatment. (A) Scheme of in vivo study. Seven week C57BL6N male mice under environmental enrichment were divided into four group for oral gavage of distilled water (DW) or Rb1 treatment of the indicated concentration for 10 consecutive days. Mice were sacrificed at day 11 and the indicated forebrains were isolated for quantitative real time PCR and western blot. (B) Real time quantitative PCR results of MBP normalized by GAPDH. One-way ANOVA with Holm-Sidak *post hoc* analysis. $^{**}P = 0.008$ (DW vs. 0.1 mg/kg Rb1). N = 4 mice per group. (C) Western blot membranes of MBP and beta-actin (b-actin). The 18.5-kDa MBP isoform, a predominant MBP isoform in adult myelin [56] was examined. Another MBP isoform, 17-kDa is also indicated. Distinct lanes indicate different mice. 30 μg protein in 10 μl is loaded per lane. (D) Western blot analysis of MBP normalized by b-actin. One-way ANOVA with Holm-Sidak *post hoc* analysis. $^{*}P = 0.032$ vs. 0.1 mg/kg Rb1, $^{*}P = 0.043$ vs. 1 mg/kg Rb1, $^{*}P = 0.045$ vs. 5 mg/kg Rb1. N = 3-4 mice per group. The graph bars indicate mean ± standard deviation. MBP, myelin basic protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ANOVA, analysis of variance.

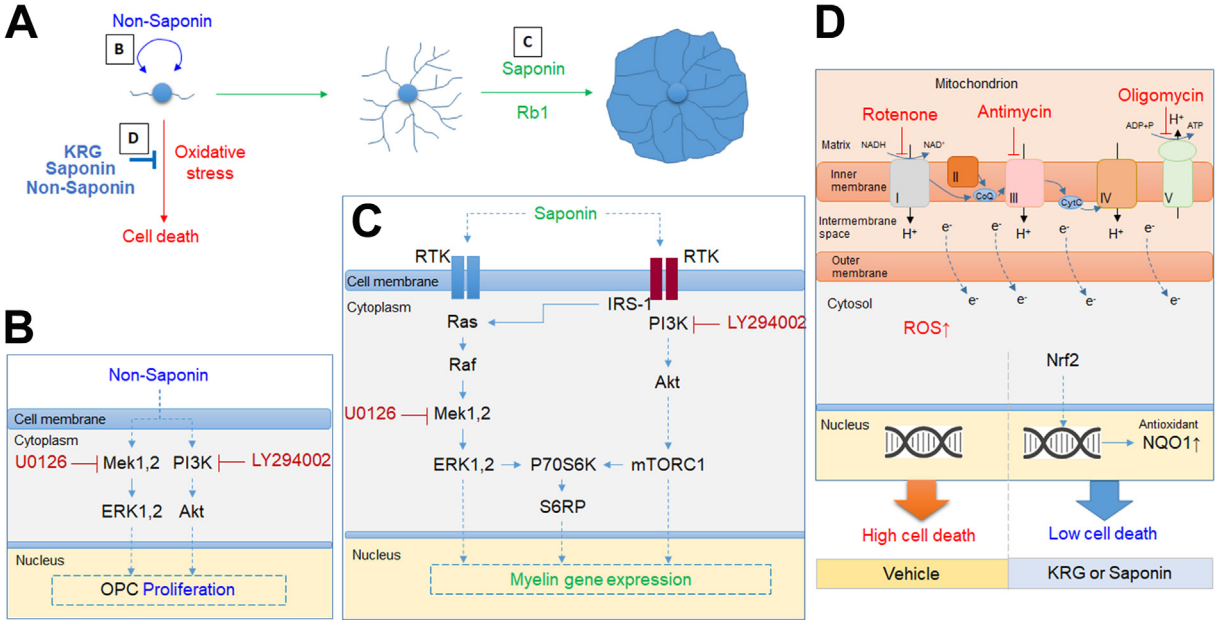

Fig. 6. Schematic explanation of the current results. (A) The non-saponin KRG fraction increased OPC proliferation, while the saponin fraction enhanced OL differentiation and membrane size. Among 11 saponin-derived ginsenosides tested, Rb1 significantly increased OL membrane size. All KRG-derived components reduced OPC death induced by oxidative stress. (B) The non-saponin fraction induced OPC proliferation, (C) while the saponin fraction induced differentiation. Both effects were mediated by ERK and AKT signaling pathways. (D) Inhibition of the mitochondrial respiratory chain reaction increases cell death potentially due to oxidative stress. The KRG total extract and the saponin fraction facilitate expression of the antioxidant NQO1, which may contribute to reduce oxidative stress-induced cell death. KRG, Korean Red Ginseng; OPC, oligodendrocyte precursor cell; OL, oligodendrocyte; ERK, extracellular regulated kinase; NQO1, NAD(P)H quinone oxidoreductase 1.

Declaration of competing interest

The authors declare no conflict of interest.

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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.2021.04.007.

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