Ginsenoside Re exhibits neuroprotective effects by inhibiting neuroinflammation via CAMK/MAPK/NF-κB signaling in microglia

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Abstract. Ginsenoside Re (G-Re) is a panaxatriol saponin and one of the pharmacologically active natural constituents of ginseng (*Panax ginseng* C.A. Meyer). G-Re has antioxidant, anti-inflammatory and antidiabetic effects. The present study aimed to investigate the effects of G-Re on neuroinflammatory responses in lipopolysaccharide (LPS)-stimulated microglia and its protective effects on hippocampal neurons. Cytokine levels were measured using ELISA and reactive oxygen species (ROS) levels were assessed using flow cytometry and fluorescence microscopy. Protein levels of inflammatory molecules and kinase activity were assessed by western blotting. Cell viability was assessed by MTT assay; apoptosis was estimated by Annexin V apoptosis assay. The results revealed that G-Re significantly inhibited the production of IL-6, TNF-α, nitric oxide (NO) and ROS in BV2 microglial cells, and that of NO in mouse primary microglia, without affecting cell viability. G-Re also inhibited the nuclear translocation of NF-κB, and phosphorylation and degradation of IκB-α. In addition, G-Re dose-dependently suppressed LPS-mediated phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase (CAMK)2, CAMK4, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinases (JNK). Moreover, the conditioned medium from LPS-stimulated microglial cells induced HT22 hippocampal neuronal cell death, whereas that from microglial cells incubated with both LPS and G-Re ameliorated HT22 cell death in a dose-dependent manner. These results suggested that G-Re suppressed the production of pro-inflammatory mediators by blocking CAMK/ERK/JNK/NF-κB signaling in microglial cells and protected hippocampal cells by reducing these inflammatory and neurotoxic factors released from microglial cells. The present findings indicated that G-Re may be a potential treatment option for neuroinflammatory disorders and could have therapeutic potential for various neurodegenerative diseases.

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Introduction

Microglia are glial cells that are located throughout the brain and spinal cord. Microglia act as resident macrophages and serve a major role in immune defense and homeostasis of the central nerve system (CNS) (1). Microglia scavenge the CNS, and activated microglia phagocytose pathogens, plaques, and damaged or unnecessary neurons (2). However, over-activation of microglia can result in excessive production of proinflammatory molecules, including nitric oxide (NO) radical, reactive oxygen species (ROS), cytokines and chemokines, which may cause neuronal cell death and brain injury (3,4). Previous studies have demonstrated that microglial activation contributes to neuronal damage and the progression of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis (ALS) (5,6).

Lipopolysaccharide (LPS) is a highly conserved outer membrane component of Gram-negative bacteria, which promotes the activation of macrophages and microglial cells. In response to LPS, microglia produce a variety of inflammatory modulators, such as IL-1β, TNF-α, IL-6, NO, ROS and prostaglandins (2). A previous demonstrated that LPS binds to target cells through CD14 and Toll-like receptors 4 (TLR4) (7). Ligation of TLR4 induces recruitment of adaptor proteins and activates subsequent downstream signaling, including mitogen-activated protein kinases (MAPKs), Ca^{2+}/calmodulin-dependent protein kinases (CAMKs) and NF-κB. In unstimulated cells, NF-κB is present in the cytosol bound to IκB; however, when stimulated by factors, such as LPS, IκB is phosphorylated by IκB kinases. Subsequently, phosphorylated (p)-IκB is rapidly ubiquitinated and degraded by the 26S proteasome complex, and the free NF-κB translocates to the nucleus leading to the expression of proinflammatory molecules (7). Moreover, MAPKs and CAMKs have been reported to be associated with inflammation in the brain and glial cells (8-11).

Panax ginseng C.A. Meyer has been used as a traditional and herbal medicine in Asia and Western countries. Ginsenosides are pharmacologically active natural constituents of ginseng (12). Ginsenoside Re (G-Re) is a panaxatriol saponin and is one of the most extensively studied ginsenosides. G-Re exhibits diverse effects, including antioxidant (13-16), anti-inflammatory (17-19) and angiogenic activities (20). Furthermore, G-Re has been reported to improve cardiac function (21,22) and exert antidiabetic effects (23-26). However, to the best of our knowledge, the effects of G-Re on neuroinflammation-associated neurotoxicity have not been fully investigated. The present study investigated the effects of G-Re on the neuroinflammatory response in LPS-stimulated microglia and its protective activities on hippocampal neurons.

Materials and methods

Materials. G-Re (purity, >98%) was purchased from Ambo Institute. LPS (phenol extracted from *Salmonella enteritidis*), MTT, and poly-L-lysine (PLL) were purchased from MilliporeSigma. KN93 (CAMK inhibitor) and KN92 (inactive analog of KN93) were purchased from Cayman Chemical Company. PD98059 (ERK inhibitor) and SP600125 (JNK inhibitor) were purchased from AG Scientific, Inc. Antibodies against inducible NO synthase (iNOS; cat. no. sc-651), cyclooxygenase 2 (COX-2; cat. no. sc-19999), NF-κB p65 (cat. no. sc-372), IκB-α (cat. no. sc-371), extracellular signal-regulated kinase (ERK; cat. no. sc-94), c-Jun N-terminal kinase (JNK; cat. no. sc-571), CAMK2 (cat. no. sc-9035), p-CAMK2 (cat. no. sc-12886-R) and β-actin (cat. no. sc-47778) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against p-ERK (cat. no. 4377S), p-JNK (cat. no. 9251S), p-IκB-α (cat. no. 2859S) and CAMK4 (cat. no. 4032S) were purchased from Cell Signaling Technology, Inc. The antibody against p-CAMK4 (cat. no. A0831) was purchased from Bioss Antibodies, Inc. The antibody against TATA-binding protein (TBP; cat. no. PAB703Mu01) was purchased from Cloud-Clone Corp. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (cat. no. ADI-SAB-300-J) and anti-mouse antibody (cat. no. ADI-SAB-100-J) were purchased from Enzo Life Sciences. Anti-CD11b-APC antibody (cat. no. 17-0112-81), CM-H_2DCFDA, DMEM and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Inc. FITC Annexin V Apoptosis Detection kit I was purchased from BD Pharmingen (BD Biosciences). Mouse TNF-α (cat. no. SMTA00B) and IL-6 ELISA kits (cat. no. S6050) were purchased from R&D Systems, Inc.

Isolation of mouse primary microglia and cell culture. ICR mice (8 weeks; weight, 30-35 g) were purchased from DBL Co., Ltd. Experimental mice were housed in plastic cages and maintained at a constant temperature (25±2˚C) and humidity (50±10%) under a 12/12-h light/dark cycle. Mice were provided with free access to food and water. One male and one female mouse were mated to obtain neonates. The animal experiments in the present study were approved by the Pusan National University Institutional Animal Care and Use Committee (approval no. PNU-2020-2651; Busan, South Korea) and were conducted in accordance with the principles in the Pusan National University Institutional Animal Care and Use Committee guidelines. Mouse primary microglia were isolated as previously described (27). After 10-14 neonates (postnatal day 2-5) of ICR mice housed in the aforementioned conditions were euthanized by decapitation, primary mixed glial cell cultures from the whole brains were prepared in PLL-coated culture flasks ($3.75x10^5$ cells/ml) and maintained in DMEM containing 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine and 50 mg/ml penicillin/streptomycin at 37˚C in 5% CO_2. After 2 weeks, the culture flasks were placed on an orbital shaker at 200 rpm and 37˚C for 5 h. The cells in medium were seeded in new PLL-coated plates and incubated at 37˚C in 5% CO_2. After 2 h, unattached cells were removed and the remaining microglia were used for further studies. To monitor purity, cells ($1.25x10^5$ cells/0.5 ml) were immunostained with CD11b-APC antibody (0.06 μg/0.5 ml) for 30 min on ice in the dark and washed with cold PBS 3 times. Then, cells were resuspended in 0.5 ml PBS and analyzed by flow cytometry (BD Accuri C6 flow cytometer; BD Biosciences). >90% of cells were stained positively (data not shown). BV2 mouse microglial cells and HT22 mouse hippocampal cells were kindly provided by Professor Youn-Chul Kim (Wonkwang University, Iksan, South Korea). Cells were grown in DMEM supplemented with 5% heat-inactivated FBS and 0.1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

Neurotoxicity of microglial-conditioned medium. Mouse primary microglia ($2.5x10^5$ cells/ml) and BV2 microglial cells ($2.5x10^5$ cells/ml) were treated with 2.5, 5.5 or 7.5 μg/ml G-Re for 1 h at 37˚C and then incubated with LPS (1 μg/ml) for 24 h at 37˚C. After incubation, cells were centrifuged at 400 x g at 4˚C for 20 min to obtain the cell-free supernatant (conditioned medium). HT22 hippocampal cells ($4x10^4$ cells/ml) were serum-starved for 4 h and then treated with 50% BV2 cell-conditioned medium or primary microglia-conditioned medium and 50% fresh DMEM at 37˚C for 24 h. For controls, HT22 cells ($4x10^4$ cells/ml) were treated with 2.5, 5.5 or 7.5 μg/ml G-Re for 1 h at 37˚C and then incubated with LPS (1 μg/ml) for 24 h at 37˚C. The viability of HT22 hippocampal cells was assessed by MTT assay or Annexin V assay after incubation.

Cell viability assay. Cell viability was assessed using the MTT-based colorimetric assay. BV2 ($2.5x10^5$ cells/ml) or HT22 cells ($4x10^4$ cells/ml) were treated with MTT (50 μg/ml) for 3 h at 37˚C in 5% CO_2. After incubation, cell culture supernatant was removed and the formazan crystals produced in viable cells were solubilized with dimethyl sulfoxide. The absorbance of each well was then measured at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Cell apoptosis assay. The Annexin V apoptosis assay was conducted using flow cytometry according to the manufacturer's instructions. Briefly, following incubation with the conditioned medium or G-Re and/or LPS, HT22 cells ($4x10^4$ cells/ml) were washed with PBS and resuspended in binding buffer at a density of $1x10^6$ cells/ml. Cells were stained with Annexin V FITC (2.5 μl) at 4˚C for 15 min and propidium iodide (2.5 μl) at 4˚C for 5 min in the dark, and then analyzed by flow cytometry (BD Accuri C6 flow cytometer;

BD Biosciences) within 1 h. Data were analyzed using BD Accuri C6 software (BD Biosciences).

Measurement of nitrite concentration. To measure nitrite (an indicator of NO levels), 100 μl aliquots were removed from culture supernatant of BV2 cells ($2.5x10^5$ cells/ml) or primary microglia ($2.5x10^5$ cells/ml) and incubated with an equal volume of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4] at room temperature for 10 min. Nitrite concentration was determined by measuring the absorbance at 540 nm with a microplate spectrophotometer (Bio-Rad Laboratories, Inc.). Sodium nitrite was used as a standard.

Measurement of TNF-α and IL-6 concentration. Mouse primary microglia ($2.5x10^5$ cells/ml) or BV2 cells ($2.5x10^5$ cells/ml) were first incubated with 2.5, 5.5 or 7.5 μg/ml G-Re for 1 h at 37˚C and then treated with LPS (1 μg/ml) for 24 h at 37˚C under 5% CO_2. Subsequently, TNF-α and IL-6 levels in the culture medium were quantified using ELISA kits according to the manufacturer's instructions.

Measurement of ROS. To evaluate the levels of intracellular ROS, BV2 cells ($2.5x10^5$ cells/ml) were treated with 1 μM CM-H_2DCFDA (general oxidative stress indicator) at 37˚C under 5% CO_2 for 30 min. The cells were then harvested and washed three times with PBS, after which the fluorescence intensity was measured by fluorescence microscopy using an Axioplan 2 microscope (Zeiss GmbH) or flow cytometry (BD Accuri C6 flow cytometer; BD Biosciences). Data were analyzed using BD Accuri C6 software (BD Biosciences). DMSO (0.04%) was used as vehicle.

Western blot analysis. Cytosolic extracts were harvested in ice-cold lysis buffer (1% Triton X-100 and 1% deoxycholate in PBS). Nuclear extracts were prepared as described previously (28). Briefly, BV2 cells were washed 3 times with cold PBS and the cell pellets were suspended in hypotonic buffer (10.0 mM HEPES-KOH; pH 7.9; 1.5 mM $MgCl_2$; 10.0 mM KCl; 0.5 mM dithiothreitol; 0.2 mM PMSF) and incubated for 15 min on ice. NP-40 (0.1%) was added to the cell extract, incubated on ice for 1 min and centrifuged at 1,700 x g for 1 min at 4˚C. Following collection of cytosolic proteins from the supernatant, nuclear proteins were extracted using buffer B (20.0 mM HEPES-KOH; pH 7.9; 25% glycerol; 420.0 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM dithiothreitol; 0.2 mM PMSF) for 30 min at 4˚C with occasional vortexing. Following centrifugation at 1,700 x g for 5 min at 4˚C, supernatant was collected and stored at -70˚C. Protein content in these extracts was determined using Bradford reagent (Bio-Rad Laboratories, Inc.). The proteins (20 μg) in each sample were resolved by SDS-polyacrylamide gel electrophoresis on 10% gels and transferred to a polyvinylidene difluoride membrane. The blotted membrane was incubated with 5% skimmed milk in PBS for 1 h at room temperature and incubated with the appropriate antibodies (1:1,000) at 4˚C overnight. Subsequently, the membrane was incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (both 1:5,000) for 1 h at room temperature and the proteins were visualized using an enhanced chemiluminescence detection system (Amersham; Cytiva). Anti-β-actin was used as the loading control for cytosolic proteins and anti-TBP was used as the loading control for nuclear proteins. Quantitative image analysis was performed using ImageJ 1.38x (National Institutes of Health) and data are presented as fold of control.

Statistical analysis. All results are expressed as the mean ± SEM. Each experiment was conducted in duplicate and repeated over three times. Statistical analysis was performed using GraphPad Prism software (version 7; GraphPad Software, Inc.). All data were statistically analyzed using one-way analysis of variance followed by Tukey's multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

G-Re suppresses LPS-induced inflammatory molecules in microglia. To determine the dose of G-Re, the effect of G-Re on cell viability was examined using the MTT assay. While LPS induced a little toxicity in BV2 microglial cells, G-Re at concentrations up to 10 μg/ml exhibited no cytotoxicity in the presence or absence of LPS (Fig. 1A). Therefore, cells were treated with G-Re at concentrations <10 μg/ml in all subsequent experiments.

In our preliminary study, LPS dose-dependently induced an inflammatory response in BV2 cells (data not shown) and 1 μg/ml LPS induced a sufficient inflammatory response, which is consistent with other reports (29-31). To investigate whether G-Re could ameliorate the LPS-mediated neuroinflammatory response, the effects of G-Re on cytokine and NO production were investigated in BV2 cells. G-Re pretreatment markedly inhibited the LPS-induced secretion of IL-6, TNF-α and NO in a dose-dependent manner (Fig. 1B-D). Consistent with these results, G-Re dose-dependently reduced the protein expression levels of iNOS and COX-2 in LPS-stimulated BV2 cells (Fig. 1E and F). Moreover, G-Re inhibited NO production, and iNOS and COX-2 expression in LPS-stimulated BV2 cells in a time-dependent manner. Because preincubation of cells with G-Re for 1 and 2 h exhibited similar effects (Fig. S1), the present study pretreated cells with G-Re for 1 h in all experiments. In addition, G-Re inhibited NO production, and the expression levels of iNOS and COX-2 in LPS-stimulated mouse primary microglia in a dose-dependent manner (Fig. 1G-I). These results suggested that G-Re may inhibit LPS-induced expression of neuroinflammatory molecules in microglia without damaging the cells.

G-Re suppresses ROS production in BV2 microglial cells. To investigate the effect of G-Re on ROS production, the levels of ROS in BV2 cells were detected using CM-H_2DCFDA. Pre-incubation with G-Re significantly diminished the levels of ROS in LPS-stimulated cells, as determined by flow cytometry (Fig. 2A and B) and fluorescence microscopy (Fig. 2C), whereas G-Re did not affect basal ROS levels. These results indicated that G-Re may inhibit LPS-induced production of ROS in microglia.

G-Re inhibits LPS-induced activation of NF-κB. Since NF-κB is a major transcription factor mediating the expression of

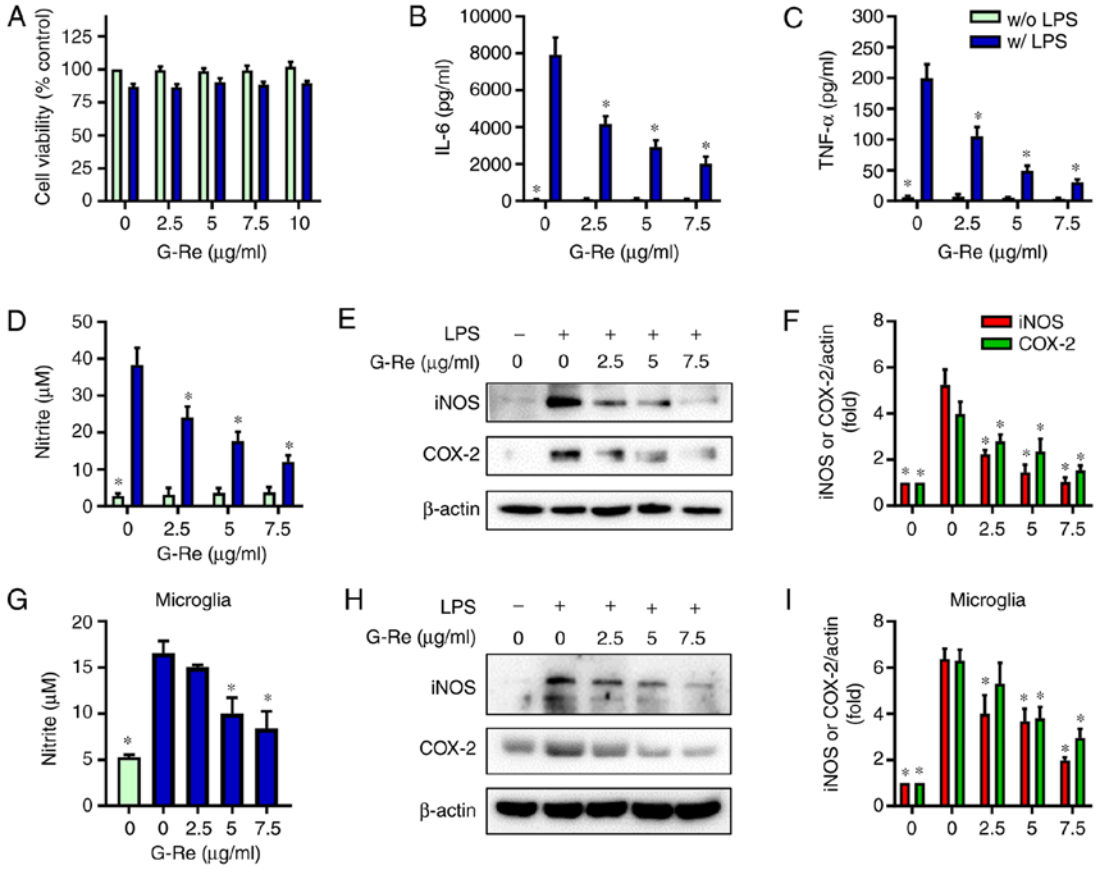

Figure 1. G-Re inhibits the production of inflammatory mediators in LPS-stimulated microglial cells. BV2 cells were treated with different concentrations of G-Re for 1 h and then incubated with or without LPS (1 μg/ml) for 24 h. (A) Cell viability was determined by MTT assay. Levels of (B) IL-6 and (C) TNF-α were measured by ELISA. (D) NO content was measured using the Griess reaction. (E) Protein expression levels of iNOS and COX-2 were detected by western blotting. (F) Relative intensity of each band (normalized to β-actin) was indicated as a ratio to the control. Mouse primary microglia were also treated as aforementioned. (G) NO content was measured using the Griess reaction. (H) Protein expression levels of iNOS and COX-2 were detected by western blotting. (I) Relative intensity of each band (normalized to β-actin) was indicated as a ratio to control. *P<0.05 vs. the group treated with LPS alone. COX-2, cyclooxygenase 2; G-Re, ginsenoside Re; iNOS, inducible NO synthase; LPS, lipopolysaccharide; NO, nitric oxide.

numerous proinflammatory genes, including IL-6, TNF-α, iNOS and COX-2, the present study investigated the effects of G-Re on NF-κB activation. As shown in Fig. 3A and B, LPS markedly increased the nuclear levels of NF-κB p65 whereas it markedly reduced the cytosolic levels of p65, indicating the nuclear translocation of p65. However, upon G-Re pretreatment, the nuclear level of p65 was decreased and cytosolic level of p65 was simultaneously increased in a dose-dependent manner. Consistent with this result, G-Re suppressed LPS-induced phosphorylation and degradation of IκB-α in a dose-dependent manner (Fig. 3A, C and D). These results suggested that G-Re inhibited LPS-induced nuclear translocation of NF-κB by preventing phosphorylation and degradation of IκB-α.

G-Re inhibits CAMKs and MAPKs involved in inflammatory mediator expression. To identify the molecular target of G-Re in the upstream signaling pathway, the effects of pharmaceutical protein kinase inhibitors of CAMK (KN93), ERK (PD98059) and JNK (SP600125) were examined. NO production, and the expression levels of iNOS and COX-2 were significantly inhibited by these inhibitors, whereas KN92, an inactive analog of KN93, exhibited no significant effects compared with those in LPS-treated cells (Fig. 4A-C). Furthermore, co-treatment with kinase inhibitors and G-Re exhibited a slightly greater inhibition of iNOS and COX-2 expression compared with treatment with either G-Re alone or kinase inhibitors alone, but there was no significant difference in NO production (Fig. S2). Moreover, G-Re suppressed LPS-induced phosphorylation of CAMK2, CAMK4, ERK and JNK in a dose-dependent manner (Fig. 4D-H). Because these kinases are activated when they are phosphorylated (32), these results indicated that G-Re inhibited activation of these kinases. Therefore, G-Re may suppress the synthesis of proinflammatory mediators by decreasing CAMK2, CAMK4, ERK and JNK activities.

Protective effects of G-Re against microglia-mediated neuronal cell death. Growing evidence has indicated that inflammatory mediators produced by microglia can induce neuronal cell death (3-6). Since the present results indicated that G-Re inhibited the expression of inflammatory mediators in LPS-induced microglia, the effects of G-Re on indirect toxicity to HT22 hippocampal neuronal cells were investigated. To accomplish this, HT22 cells were treated with BV2-conditioned medium and an MTT assay was performed to assess cell viability and an Annexin V assay was conducted to detect apoptosis. When HT22 cells were incubated with

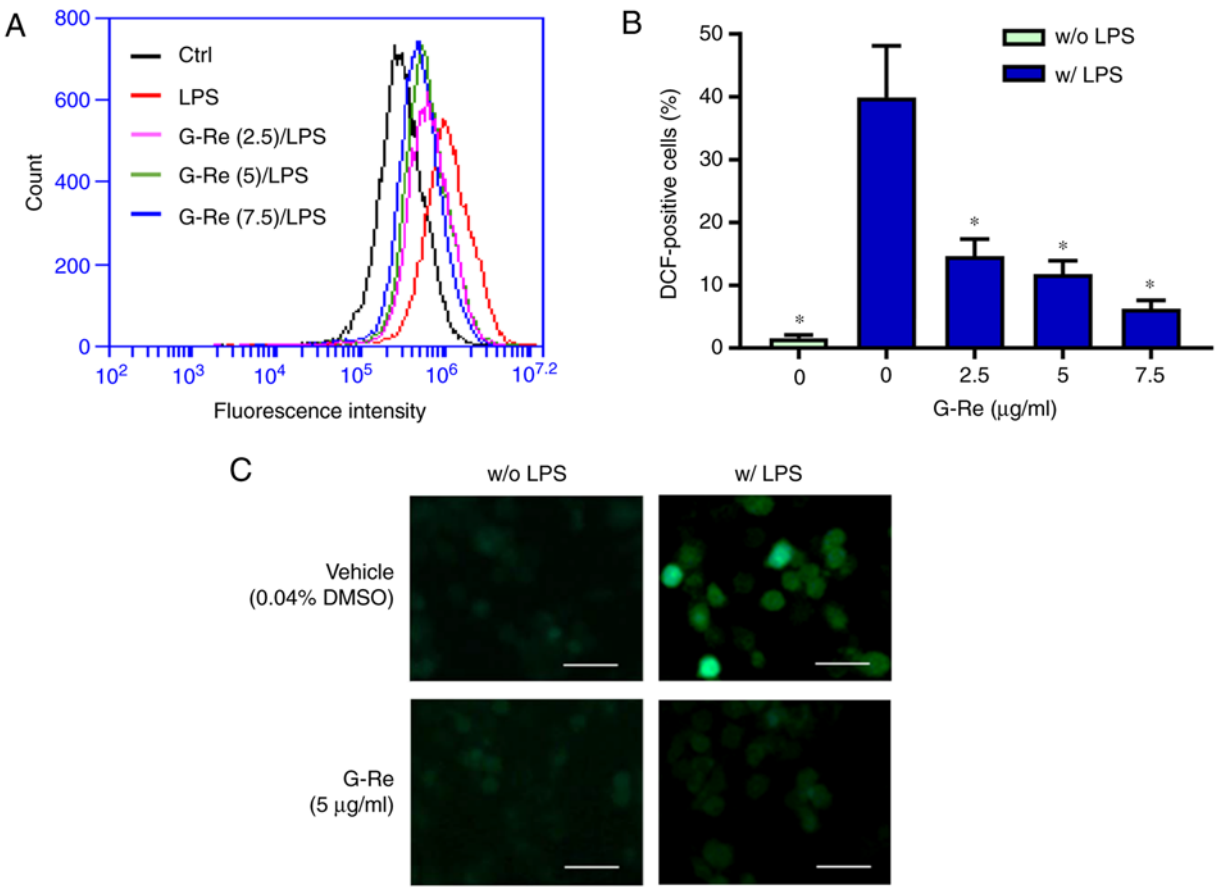

Figure 2. Effects of G-Re on LPS-induced ROS production in BV2 microglial cells. BV2 cells were treated with G-Re for 1 h and then incubated with LPS (1 μg/ml) for 30 min. DMSO (0.04%) was used as vehicle. After incubation, the cells were treated with CM-H_2DCFDA for an additional 30 min. The intracellular levels of ROS were then determined by (A and B) flow cytometry and (C) fluorescence microscopy. (A) A representative histogram of flow cytometry is presented. Scale bar, 50 μm. *P<0.05 vs. the group treated with LPS alone. G-Re, ginsenoside Re; LPS, lipopolysaccharide; ROS, reactive oxygen species.

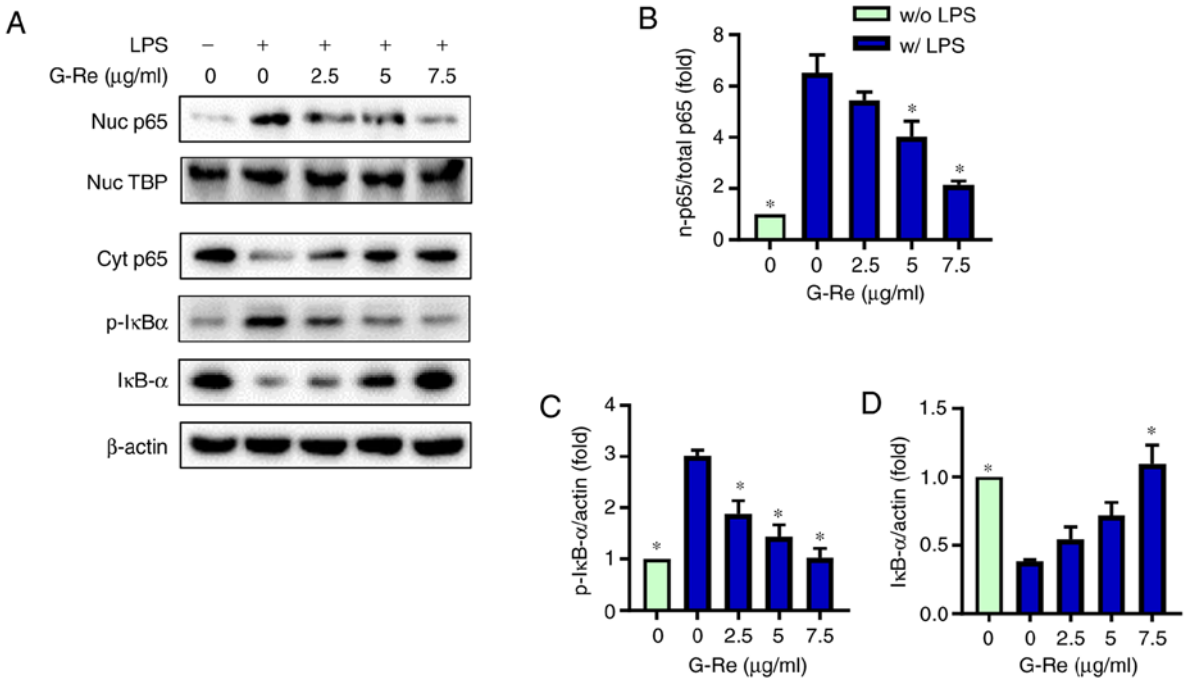

Figure 3. Inhibitory effects of G-Re on NF-κB signaling. BV2 cells were treated with various concentrations of G-Re for 1 h followed by LPS (1 μg/ml) treatment for 30 min. (A) Protein expression levels of NF-κB p65 in the nuclear and cytosolic fractions, and of IκB-α and p-IκB-α in cytosolic extracts were analyzed by western blotting. (B) Relative intensity of p65 in the nucleus to total p65 [(nuc p65/TBP)/(nuc p65/TBP)+(cyt p65/actin)] was indicated as a ratio to control. Relative intensity of (C) p-IκB-α and (D) IκB-α (normalized to β-actin) was indicated as a ratio to control. *P<0.05 vs. the group treated with LPS alone. G-Re, ginsenoside Re; LPS, lipopolysaccharide; p, phosphorylated; TBP, TATA-binding protein.

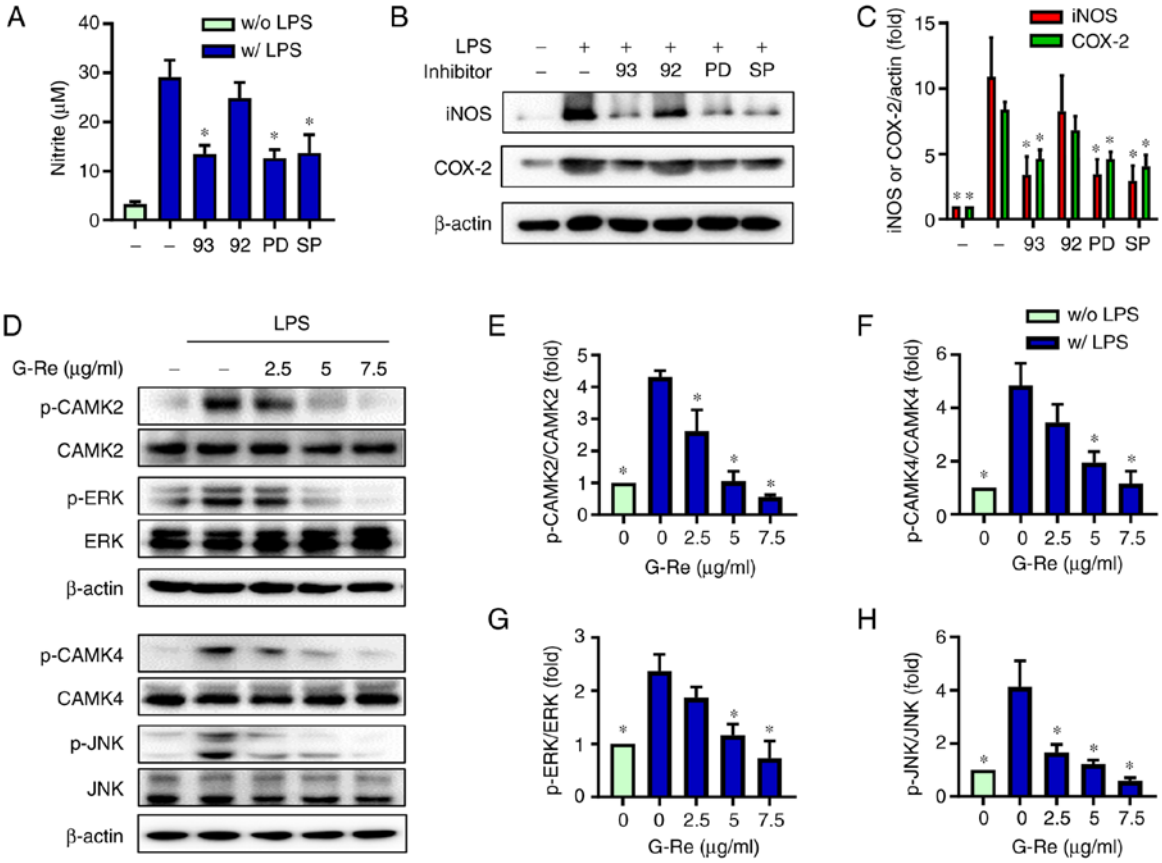

Figure 4. Effects of G-Re on LPS-induced activation of CAMKs and mitogen-activated protein kinases. BV2 cells were incubated with KN93 (5 μM), KN92 (5 μM), PD98059 (5 μM) or SP600125 (5 μM), followed by LPS (1 μg/ml) treatment for 24 h. (A) Nitric oxide content was measured, and (B) protein expression levels of iNOS and COX-2 were detected by western blotting. (C) Relative intensity of each band (normalized to β-actin) was indicated as a ratio to control. BV2 cells were treated with various concentrations of G-Re for 1 h, followed by LPS treatment (1 μg/ml) for 30 min. (D) Expression levels of unphosphorylated kinases or p-kinases were analyzed by western blotting. (E-H) Relative intensity of p-kinases (normalized to respective unphosphorylated kinases) was indicated as a ratio to control. *P<0.05 vs. the group treated with LPS alone. CAMK, Ca^{2+}/calmodulin-dependent protein kinase; COX-2, cyclooxygenase 2; ERK, extracellular signal-regulated kinase; G-Re, ginsenoside Re; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; p, phosphorylated.

the conditioned medium from LPS-stimulated BV2 cells, cell viability was markedly decreased. However, treatment of HT22 cells with the conditioned medium from BV2 cells incubated with both LPS and G-Re enhanced HT22 cell viability in a dose-dependent manner; treatment of the cells with the conditioned medium from only G-Re-treated BV2 cells exhibited no effect (Fig. 5A). Consistent with the MTT assay results, conditioned medium from LPS-treated BV2 cells led to marked apoptotic cell death [both early (Annexin V-positive and PI-negative fraction) and late (Annexin V-positive and PI-positive fraction) apoptosis], whereas application of the conditioned medium from LPS + G-Re-treated BV2 microglial cells decreased Annexin V-positive cells (Fig. 5C and D). Notably, direct stimulation of HT22 neuronal cells with either LPS alone or both LPS and G-Re had no significant effect on cell viability (Fig. 5B) and apoptotic cell death (Fig. 5C and D). Furthermore, similar results were obtained from the MTT assay following treatment of cells with the conditioned medium from mouse primary microglial cells (Fig. 5E). Overall, these results suggested that factors released from LPS-treated microglial cells may induce neuronal toxicity and that G-Re could protect HT22 hippocampal cells by reducing these factors released from microglial cells.

Discussion

The present study investigated the anti-inflammatory effects of G-Re on LPS-stimulated microglial cells. The results revealed that G-Re pretreatment significantly inhibited the LPS-mediated production of IL-6, TNF-α, NO and ROS, and the expression levels of iNOS and COX-2. IL-6 and TNF-α are proinflammatory cytokines, which are known to be involved in the pathogenesis of various inflammation-related diseases. IL-6 administration has been shown to cause mechanical allodynia and thermal hyperalgesia (33). In addition, IL-6 may impair oligodendrocyte regeneration and induce demyelination (34). Moreover, chronic microglial activation in GFAP-IL6 mice contributed to the age-dependent loss of cerebellar volume and impairment in motor function (35). IL-6 levels have been reported to be increased in the cerebrospinal fluid of patients with viral meningitis, encephalitis, systemic lupus erythematosus and stroke (36-39). IL-6 may also enhance neuronal damage induced by β-amyloid peptide in cultured rat cortical neurons (40). Furthermore, the TNF-α protein synthesis inhibitor has been shown to restore neuronal function and reverse cognitive deficits induced by chronic neuroinflammation (41), and it has been demonstrated that inhibition of TNF-α can lead to favorable outcomes in Alzheimer's disease (42).

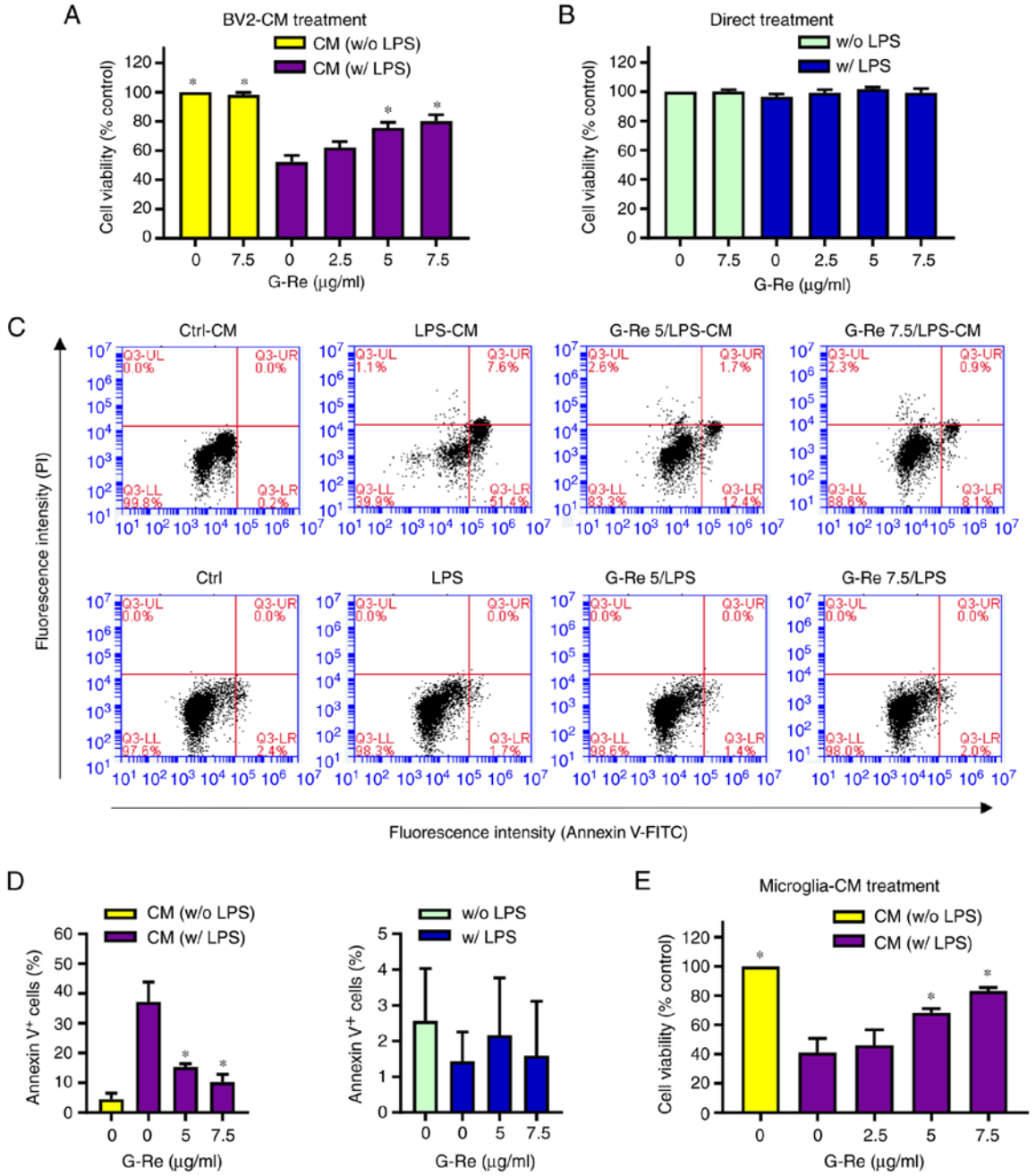

Figure 5. Effects of G-Re on inflammation-induced neurotoxicity of HT22 hippocampal cells. (A) BV2 cells were incubated with LPS (1 μg/ml) in the presence or absence of G-Re for 24 h, and then HT22 cells were treated with BV2-CM. After 24 h, the viability of HT22 cells was estimated by MTT assay. (B) HT22 cells were incubated with LPS (1 μg/ml) in the presence or absence of G-Re for 24 h, and cell viability was estimated by MTT assay. (C and D) HT22 cells were treated with BV2-CM as aforementioned, or with LPS (1 μg/ml) in the presence or absence of G-Re for 24 h, and an apoptosis assay was performed. (C) Representative flow cytometry plots and (D) percentage of Annexin V-positive cells were shown. (E) Mouse primary microglia were incubated with LPS (1 μg/ml) in the presence or absence of G-Re for 24 h, and then the microglia-CM was added to HT22 cells. After 24 h, the viability of HT22 cells was estimated by MTT assay. *P<0.05 vs. the group treated with LPS alone. CM, conditioned medium; G-Re, ginsenoside Re; LPS, lipopolysaccharide; PI, propidium iodide.

ROS in the brain are involved in the development of oxidative neuronal damage and progression of neurodegenerative diseases (43,44). In addition, high amounts of NO produced by iNOS in activated microglial cells are considered to cause neuronal cell damage and lead to neurodegeneration (45,46), because neurons are notably sensitive to NO-induced cell death (47). Furthermore, NO reacts with superoxide to produce peroxynitrite, which is a powerful oxidant and a potent inducer of cell death (48). Thus, suppression of IL-6, TNF-α, NO and ROS production by G-Re in microglial cells may contribute to reduced neurodegeneration and neuroinflammation. In agreement with the present results, previous studies have reported that G-Re exhibits anti-inflammatory effects (17-19). G-Re has been shown to inhibit the release of histamine from human mast cells, and the expression of IL-1α, IL-8, IL-10 and RANTES in a human alveolar cancer cell line (17).

NF-κB is a vital transcription factor for several genes, which is involved in regulating immune and inflammatory responses, such as the expression of cytokines, iNOS and COX-2 (49). Improper regulation of NF-κB has been shown

to be directly involved in a wide range of human disorders, including neuroinflammatory and neurodegenerative diseases (50,51); therefore, the development of drugs that regulate NF-κB is considered a promising strategy for therapeutic manipulation of inflammatory disease (52). The present study revealed that G-Re significantly inhibited the nuclear translocation of NF-κB and the degradation of IκB-α. Moreover, G-Re effectively inhibited the phosphorylation, and thus the activation, of CAMK2, CAMK4, ERK and JNK. Thus, these results suggested that G-Re may inhibit the expression of inflammatory mediators, such as IL-6, TNF-α, iNOS and COX-2 via blocking CAMK2/CAMK4/ERK/JNK/NF-κB signaling in microglial cells. To the best of our knowledge, the present study is the first to demonstrate that G-Re suppressed CAMK2/CAMK4 activities.

Brain inflammation is considered to promote neurodegenerative diseases and cognitive dysfunction. Notably, an increasing number of reports have shown that systemic or intracerebroventricular administration of LPS can lead to β-amyloid generation and memory deficiency (53-55). LPS-induced neuroinflammation may also be accompanied by hippocampal neuronal death and microglia activation. During neuroinflammation, microglia are activated and release proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. The neuroinflammatory molecules released by activated microglial cells can induce indirect neuronal toxicity, which may also contribute to neurodegenerative disorders (56-59). The results of the present study demonstrated that apoptosis of HT22 hippocampal neuronal cells was induced following treatment with the conditioned medium from LPS-stimulated microglia, which is hypothesized to secrete neurotoxic molecules. By contrast, G-Re significantly attenuated HT22 cell death, and this protective effect of G-Re on indirect neuronal toxicity may be due to its ability to reduce the secretion of these neurotoxic molecules from microglia. Neither LPS nor G-Re exhibited any direct effects on the viability of HT22 cells after treatment for 24 h; therefore, it may be hypothesized that it is not the direct effects of G-Re that inhibit HT22 cell death, but the effects of G-Re on the microglia that suppress HT22 cell death. These results suggested that G-Re could potentially ameliorate various neuroinflammatory and neurodegenerative diseases. This hypothesis is supported by a study demonstrating that G-Re attenuated neuroinflammation in a symptomatic ALS animal model generated using human-superoxide dismutase 1 transgenic mice (60). This previous study demonstrated that administration of G-Re enhanced the number of motor neurons and reduced the number of microglia.

In conclusion, the present study demonstrated that G-Re suppressed LPS-induced overproduction of pro-inflammatory mediators, including IL-6, TNF-α, NO and ROS, in microglial cells. In addition, G-Re significantly inhibited LPS-induced activation of NF-κB p65, CAMK2, CAMK4 and MAPKs, such as ERK and JNK. Furthermore, G-Re attenuated HT22 hippocampal cell death induced by neurotoxic molecules released from activated microglia. These findings suggested that G-Re may have therapeutic potential for the treatment of neuroinflammatory and neurodegenerative diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

IM, JHK and JES performed the experiments. YK and IM analyzed the data. YK and IM confirm the authenticity of all the raw data. YK wrote the manuscript and conceptualized the study design. YK and JHK revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments in the present study were approved by the Pusan National University Institutional Animal Care and Use Committee (approval no. PNU-2020-2651).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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