INTRODUCTION

The antistress effects of ginseng are well known, and its active ingredients have been identified as ginsenosides [1,2]. Immobilization (IMO) stress-induced polyamine levels were decreased by ginsenosides Rb1 and Rg3, and by ginseng total saponin in mice brains [3]. Rb1, the main ingredient of ginseng, has a neuroprotective effect against glutamate-induced neurotoxicity [4,5], ischemia [6], neurodegeneration [7], and seizures [8]. Rg3 in fermented red-ginseng has anti-stress [4] and neuroprotective effects [9].

The structure of ginsenoside is similar to that of steroids [10]. Ginsenoside Rh1 and Rb1 showed estrogen receptor (ER) activation and ER-dependent action, respectively, in the mammary gland and uterus [10]. Ginseng also activates ER in breast cancer cells in vitro but not in vivo [11]. Although ER was activated in endothelial cells by protopanaxadiol and protopanaxatriol ginsenosides in vitro [12], ER is not regulated by Rh2 in brain astrocytes [13]. These results indicate that the effect of ginseng on the brain should be further investigated.
Membrane lipids in the brain can undergo oxidative damage upon physical stress such as a cold swim, electric foot shock, and IMO [14]. However, investigation of the anti-stress effects of ginseng and ginsenosides has been focused on biochemical parameters such as plasma interleukin (IL)-6 [15], plasma cholesterol, glucose, serum corticosterone [16], in vitro free radical scavenging activity [17], proinflammatory cytokines (tumor necrosis factor [TNF]-α, IL-1β, and IL-6) [18], and malondialdehyde levels [19]. Rg1 also prevented glutathione reduction and superoxide dismutase activation induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the brains of mice [20]. However, ginseng increases the release of cytokines and the expression of toll-like receptor 4 [21]. Our previous study showed that IMO as well as in vitro oxidative stressors such as H2O2, acrylamide, and an endoplasmic reticulum stressor tunicamycin significantly induced peptidyl arginine deiminase type IV (PADI4) gene. Moreover, pretreatment with red ginseng (RG, steamed and dried ginseng) derepressed ERβ, which subsequently represses induction of PADI4. Consistently, RG pretreatment inhibits the production of cyclooxygenase 2 and malondialdehyde demonstrating that RG protects the brain from cell death by repressing PADI4 via ERβ stimulation [22]. However, the mechanism whereby RG affects gene expression of TNF-α and downstream nuclear factor (NF)-κB remains unknown. Here we found that RG repressed PADI4 via TNF-α convertase (TACE) and NF-kB in brain cells, thus preventing production of reactive oxygen species (ROS) and subsequently protecting brain cells from apoptosis.

MATERIALS AND METHODS

Cells, animals, immobilization stress, red ginseng, and treatment

Human neuroblastoma SK-N-SH cells (ATCC HTB-11; American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 (Lonza, Walkersville, MD, USA) media containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (10,000 U penicillin/mL, 10,000 mg streptomycin/mL), 1 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L bicarbonate, 2 mM L-glutamine at 37°C, and 5% CO2. Rat neuroglioma C6 (ATCC CCL-107, American Type Culture Collection) was cultured in Dulbecco’s modified Eagle medium containing 10% FBS and 2% penicillin-streptomycin. RG stock was prepared at 10 mg/mL in phosphate buffer saline (PBS, pH 7.4), diluted with RPMI 1640 media containing 10% FBS and 2% penicillin-streptomycin to 1 mg/mL just prior to use and sterilized by filtration with a 0.22 μm bottle top filter (Nalgene, Rochester, NY, USA). Male ICR mice (20 to 25 g) were housed in a temperature-controlled environment (temperature 21±2°C, humidity 60±10%) and 12-hour dark: 12-hour light cycle under conditions where food and water were freely available. All experiments conformed to the animal care guidelines of the Korean Academy of Medical Sciences, and all efforts were made to minimize animal suffering. Stress procedures were approved and monitored by the ethical committee of Sungkyunkwan University. For stress experiments, mice were immobilized for 30 or 45 min in a tightly fitted, 50 mL conical tube. At the end of the stress period, the mice were sacrificed by cervical dislocation, and the brain was rapidly removed and frozen. Animals that were set free in their home cage in the absence of any stressors served as controls (the normal control group). Mice were divided into three groups: non-treated (no stress), stress + no treat, and stress + RG. The non-treated group was used as a control. The stress + no treat group was administrated only PBS. Lastly, the stress + RG group was administrated RG orally (RG extract; Korea Ginseng Corporation, Daejeon, Korea) twice a day for one week. The mice began fasting on the evening of the 7th day and were sacrificed 3 h after the last administration of RG on the morning of the 8th day.

For in vitro experiments, a filtered RG solution was further diluted with RPMI 1640 media to 1 mg/mL prior to RG treatment. In addition, cells were treated with RG for 48 h.

Determination of nitric oxide and reactive oxygen species

For ROS determination, 2×104 SK-N-SH cells were cultured overnight, and treated with 1 mg/mL of RG for 48 h or RPMI 1640 media alone (control). After treatment with 0.5 mM H2O2 for 20 min, cells were washed with PBS, and the intracellular accumulation of ROS was measured using the fluorescent probe H2DCFDA. At the end of the treatments, cells were loaded with 20 μM H2DCFDA and incubated at 37°C for 30 min in the dark. Cells were then collected and resuspended in PBS. The fluorescence was measured immediately by flow cytometry (Biorad, Hercules, CA, USA).

For nitric oxide (NO) determination, C6 cells were pretreated with 0.5 mg/mL of RG for 24 h, and then 1 μg/mL of lipopolysaccharides (LPS; Sigma, St. Louis, MO, USA) was added. The amount of NO in cell su-
pernatants was measured spectrophotometrically by the Griess reaction. Samples were supplemented with 276 mU of nitrate reductase and 40 μM NADPH and then allowed to react with the Griess reagent (aqueous solution of 1% sulfanylamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H₃PO₄) in order to form a stable chromophore absorbing at a wavelength of 546 nm.

**Purification of total RNA**

Total RNA from mouse brain or tissue cultured cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. RNA quality was measured spectrophotometrically.

**Reverse transcriptase polymerase chain reaction**

Total RNA was reversely transcribed to complementary DNA by M-MLV reverse transcriptase (RT) (RexGene Biotech, Ochang, Korea). All polymerase chain reaction (PCR) primer pairs were designed for mRNA sequencing within 200 bp; p53, 5'-CTGAGG TTGGTCTGACTGTACCAC-CATCC3' (forward) and 5'CTCATTCAGCTCTCGGAACATCTCGA-AGCG3' (reverse); β-actin, 5'-TGG AAT CCT GTG GCA TCC ATG AAA-3' (forward) and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3' (reverse). Relative quantification of select mRNA was performed on 20 µl of cDNA using StepOne (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The RT PCR conditions were optimized to comprise an initial denaturation step of 10 min at 95°C, followed by 35 cycles of 95°C for 10 s, 55°C for 15 s and 72°C for 20 s. Statistical analysis was by analysis of variance between groups (ANOVA).

**Western blot analysis**

Cells were collected by centrifugation after washing with PBS, and resuspended in 50 mM Tris-Cl pH 7.4, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride and 1X protease cocktail inhibitor. Cells were lysed by sonication and cell lysates were harvested after centrifugation at 12,000 rpm, 4°C for 15 min. The amount of protein was determined by Bradford assay and 30 to 40 µg of protein was used for Western blot. Proteins separated by 10% or 15% SDS-polyacrylamide gel electrophoresis were electroblotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was then incubated in blocking buffer (7% skim milk, in TPBS [PBS, 0.1% Tween 20]) at room temperature on a shaking incubator for more than 1 h. After washing 3 times with TPBS, the membrane was incubated in a primary antibody (1:1,000) for 1 h at room temperature with primary antibodies PADI4, TACE (Abcam, Cambridge, England), Bel-2, caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Sigma), or p65 NF-κB (Cell Signaling, Beverly, MA, USA). After extensive washing, the membrane was incubated with a secondary antibody (horseradish peroxidase-conjugated anti-IgG antibody, 1:5,000 or 1:10,000 anti-rabbit, anti-mouse; Sigma) for 1 h. Bands were detected with Power Optic-ECL western blotting detection reagent (iNtRON Biotech, Seongnam, Korea).

**Immunohistochemistry**

In order to determine the level of TACE, immunohistochemistry was performed on mouse formalin-fixed, paraffin-embedded 3-μm-thick sections with Discovery XT (Ventana Medical Systems, Tucson, AZ, USA). TACE antibody (Abcam) was used at a 1:100 dilution. For immunofluorescence, anti-red fluorescent protein antibody (Rockland, Gilbertsville, PA, USA) was used at 1:200 followed by a Alexa Fluor 488 anti-rabbit secondary antibody (Invitrogen) according to the manufacturer’s protocol.

**Transfection**

To knock down PADI4 expression by siRNA, 100 nM of siRNA (Genolution Pharmaceuticals, Seoul, Korea) was added to 250 μL of serum-free RPMI 1640 media without antibiotics, to which 5 μL of transfection reagent (TransIT-TKO; Mirus, Madison, WI, USA) had been added and incubated for 20 min at room temperature. SK-N-SH cells (3×10⁵ cells/well) were cultured in 6-well plates overnight, washed once with serum-free media, and replaced with 1 mL of serum-free media containing antibiotics. Subsequently, transfection reagent containing siPADI4 was added to the cell culture and incubated for 24 h. As a control, cells were transfected with mock siRNA. After 24 h incubation, siRNA was removed and replaced with new complete media containing 1 mg/mL RG (cells in RPMI 1640 media alone were used as a control) for 48 h. Subsequently, cells were washed with PBS twice, and exposed to 0.5 mM H₂O₂ for 2 h followed by washing and lysis.

**Luciferase reporter gene assay**

NF-κB was determined by the luciferase reporter gene
assay according to the manufacturer’s protocol. Briefly, SK-N-SH cells were seeded in a 12-well plate with $1 \times 10^5$ cells per well in 1 mL of RPMI 640 media. Then, cells were transfected with 1 μg of NF-xB plasmid (pGL3-NFκB) with the Renilla luciferase reporter mRNA. After 24 h, cells were treated with RG for another 24 h followed by treatment with 0.1 mM H$_2$O$_2$ for 4 h. Then the cells were collected in a passive lysis buffer (Promega, Madison, WI, USA), and the supernatant was used for determination of luciferase activity with the reporter assay system (Promega).

**Statistical analysis**

Data were analyzed by ANOVA followed by Dunett’s $t$-test for comparisons between groups. Significance was accepted when $p<0.05$ ( *$p<0.05$, **$p<0.01$, and ***$p<0.001$). Data are expressed as median±standard deviation for three to five independent experiments.

**RESULTS**

**Reversion of cell death-associated gene expression by red ginseng**

Previously, we demonstrated that RG represses PADI4 expression as well as cell death, and induced Bcl-2, an anti-apoptotic factor, in vivo. Moreover, RG repressed active p53 expression after oxidative stress in vitro [22]. However, other apoptosis-related factors such as caspase-3 and p53 were not examined in vivo. To confirm the anti-apoptotic nature of RG, we investigated these two markers after IMO stress. When mice were subjected to IMO stress, the level of p53 mRNA was significantly repressed. However, RG pretreatment reversed this repression back to normal (Fig. 1A). To corroborate anti-apoptosis at the protein level, we determined the expression levels of representative apoptotic/anti-apoptotic markers caspase-3 and Bcl-2 by Western blot. Consistently, RG pretreatment increased Bcl-2 and repressed caspase-3 (Fig. 1B) indicating that RG could reverse IMO-induced apoptosis.

**Repression of tumor necrosis factor-α convertase by red ginseng**

System biology analyses previously showed that IMO stress up-regulated immunereseponse-induced genes, and TNF-α was found at the center of the network [22]. Moreover, TNF-α was significantly induced by IMO stress but RG administration reversed TNF-α induction [22]. Normally, TNF-α is bound on the cell membrane in an inactive form, and is cleaved by a TACE (synonyms: ADAM-17 [a disintegrin and metalloproteinase 17], CD156b, cSVP and MGC71942) to be released in an ac-
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Repression of soluble form [23]. To further elucidate the function of RG in IMO-affected mice, the TACE level in the brains of IMO-stressed mice was determined by immunohistochemistry. TACE was induced by IMO specifically in the medulla and the spinal cord (Fig. 2). In addition, LPS, a well-known inducer of TNF-α, also induced TACE. However, pre-administration of RG repressed induction of TNF-α (Fig. 2) indicating that TNF-α induction in the IMO-stressed brain could be ascribed to TACE activation.

TNF-α and ROS activate c-Jun N-terminal kinases (JNK) [24], and JNK further activates p53 as well as other apoptotic factors [25]. Therefore, to further investigate how RG affected cell death after PADI4 induction, SK-N-SH cells were transfected with siPADI4 prior to H₂O₂ stress, and expression of TACE was determined by Western blot. As a control, siPADI4 was shown to repress PADI4 expression (Fig. 3). Interestingly, expression of TACE was not affected by siPADI4 in the non-RG-treated stressed condition. However, RG pretreatment repressed expression of TACE compared to the mock transfected control under oxidative stress conditions (Fig. 3). These results indicated that oxidative stress induced PADI4, which subsequently activated TACE. However, RG repressed PADI4 induction, suggesting that PADI4 may be an upstream regulator of TACE.

**Inhibition of nuclear factor-κB by red ginseng**

To further examine whether RG could inhibit inflammation, we investigated induction of NF-κB by oxidative stress [24] using a reporter gene assay. We found that exposure to H₂O₂ significantly increased the NF-κB level compared to non-oxidative conditions. However, RG pretreatment significantly repressed NF-κB induction (Fig. 4A). To confirm repression of NF-κB induction by RG, translocation of NF-κB from the cytosol to the nucleus was determined. After exposure to H₂O₂, the NF-κB level in the nucleus was significantly increased whereas the NF-κB level was significantly decreased by RG pretreatment (Fig. 4B, C). These results indicated that translocation of NF-κB to the nucleus as well as NF-κB induction were significantly repressed by RG treatment.

**Anti-inflammatory effect of red ginseng**

Since NF-κB induction could produce ROS [24], we
examined effect of RG on ROS production and NO release to further corroborate repression of NF-κB. When SK-N-SH cells were exposed to H₂O₂, the level of ROS increased significantly. However, RG pretreatment significantly repressed ROS induction (Fig. 5A). To confirm repression of ROS production by RG, LPS-induced NO production was assessed. The results showed that RG pretreatment significantly reduced NO production (Fig. 5B).
release was examined. When C6 glioma cells were treated with LPS, the NO level increased in a time-dependent manner. Moreover, RG pretreatment significantly inhibited the level of release of LPS-induced NO (Fig. 5B) suggesting that RG repressed LPS-induced NO release. These results demonstrated that RG inhibited the expression of genes involved in inflammation and oxidative stress thereby decreasing production of oxidative compounds and subsequently protecting the brains of mice from oxidative damage.

**DISCUSSION**

IMO stress was previously shown to upregulate the immune response-associated gene TNF-α in the brains of mice [22]. Moreover, restraint stress increases expression of the inducible isoform of NO synthase (iNOS) in rat brains, and iNOS inhibitor aminoguanidine protects from stress-induced pathophysiology [26]. However, how TNF-α expression could be controlled by RG remained unknown. In this study we demonstrated that RG inhibited PADI4 via TACE expression followed by inflammation and apoptosis.

Once immune-response associated genes are induced, they could disrupt homeostasis and eventually result in a disease state. Our previous result demonstrated that cell death-associated genes were induced by IMO stress [22]. Moreover, IMO stress increased apoptosis [27]. Apoptosis is induced by activation of caspases such as caspase-3 [28]. However, in this study we showed that RG repressed caspase-3 expression and increased anti-apoptotic Bcl-2 and p53, indicating that RG repressed apoptosis. RG also repressed TACE expression in vivo (Fig. 2). Moreover, siPADI4 repressed both TACE and PADI4 expression collaterally (Fig. 3), indicating that PADI4 induced TACE expression in the presence of RG. Consistently, RG could repress NF-κB induction as well as NF-κB translocation under oxidative stress conditions (Fig. 4), which subsequently repressed ROS and NO production (Fig. 5). Since restraint stress increases expression of the iNOS in rat brains, and iNOS inhibitor aminoguanidine protects from stress-induced pathophysiology [26], our results suggest that RG can modulate gene expression via various activities comprising PADI4, TACE, NF-κB, and ROS followed by a net reduction in cell death.

Physical, psychological or mixed stress in the brain triggers inflammatory responses including the release of several inflammatory cytokines, free radicals, prostanooids and transcription factors, activation of neutrophils, and the release of protein oxidation marker myeloperoxidase culminating in damaged brain cells [29]. In this study, we showed that IMO stress could induce inflammatory responses in the brain, leading to tissue damage. Interestingly, anti-inflammatory pathways are also activated in the brain in response to stress, to defend against inflammation-induced damage, thus resulting in dualizing pro-and anti-inflammatory responses [29]. RG may thus also induce an anti-inflammatory response to dampen the pro-inflammatory response incurred by oxidative stress. Further studies will be needed to elucidate the underlying mechanism of our observations.

Transcription in eukaryotic cells can be regulated at the post-translational level by histone modifications including methylation, phosphorylation, and ubiquitination, and histone arginine methylation is catalyzed by protein arginine methyltransferases [30]. PADI4 demethylates histone arginine at the p21 promoter region [31]. PADI4 thus represses the expression of genes induced by estrogen and retinoic acid receptors [32,33], serves as a p53 corepressor and represses the expression of p53 target genes p21/WAF1/CIP1 [31]. Although we demonstrated that siPADI4 attenuated cell death [22] and RG pretreatment increased p53 expression (Fig. 1A), we cannot exclude other mechanisms of anti-apoptosis. Our system biology analysis and network analyses showed that IMO stress shows significantly higher induction of cell death-

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**Fig. 6.** Red ginseng (RG) inhibits apoptosis in an oxidatively-stressed brain via tumor necrosis factor (TNF-α) convertase (TACE). Oxidative stress induces peptidyl arginine deiminase type IV (PADI4), which activates TACE and nuclear factor (NF-κB) translocation, triggering cues of inflammation as well as production of reactive oxygen species (ROS). Activation of PADI4 and TACE results in apoptosis. RG represses TACE via PADI4, thereby inhibiting apoptosis in the brain cells.
associated genes such as HOXA5 (apoptosis), ITGB3BP (killing and adhesion), and LGALS3BP (cell death) than other genes. Moreover, RG pretreatment up-regulates apoptosis-associated genes including caspase, CASP9, and HOXA5, yet RG pretreatment did not result in increased cell death. Therefore, RG may also induce other as-yet-unknown inhibitors of apoptosis proteins such as Bruce, cIAP1, cIAP2, Survivin (BIRC5), or XIAP [34-36] alone or in combination, followed by inhibition of apoptosis. In this case, RG would show anti-apoptotic activity via caspase-independent pathways. Further studies are warranted to determine which mechanism is involved.

Taken together, we found that oxidative stress by IMO induced PADI4, which activated TACE and subsequently the release of TNF-α and NF-kB activation/translocation, which further induced inflammation and ROS secretion. In contrast, RG repressed PADI4 expression, thus reversing stress-induced gene expression and subsequently inhibiting apoptosis by repressing TACE and NF-kB activation/translocation, and by repressing ROS and NO production (Fig. 6).

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