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Contribution of ginsenoside Re to cellular redox homeostasis via upregulating glutathione and superoxide dismutase in HaCaT keratinocytes under normal conditions

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Ginsenoside Re (Re) is one of the main ginsenosides which are known to be responsible for diverse pharmacological properties of ginseng, widely used as a dietary supplement and a general tonic. The present work was undertaken to evaluate the antioxidative property of Re by analyzing reactive oxygen species (ROS), nitric oxide (NO), pro-matrix metalloproteinase-2 (proMMP-2) and -9 (proMMP-9), total glutathione (GSH) and superoxide dismutase (SOD) in normal, unstressed HaCaT keratinocytes. When HaCaT cells were subjected to Re, Re suppressed the ROS and NO levels in a concentration-dependent manner. Re at concentrations used exhibited no cytotoxicity on the cellular viabilities of HaCaT cells. It was also able to attenuate proMMP-2 and -9 at both activity and protein levels. On the contrary, Re was capable of enhancing the total GSH and SOD activity levels. The findings suggest that Re has an antioxidative property through the upregulation of some antioxidant components, including total GSH and SOD, in HaCaT keratinocytes, which then can play its underlying role in maintaining the cellular redox homeostasis.

1. Introduction

Ginseng, traditionally referring to the dried roots of *Panax ginseng* C.A. Meyer (Araliaceae), has been used as a general tonic in oriental medicine for a long time. Ginsenosides are active ingredients responsible for most currently identified pharmacological efficacies of ginseng. Although diverse ginsenosides are categorized into four major classes depending on the chemical structures of their aglycones called sapogenins, most glycosides are comprising dammarane sapogenins, divided into protopanaxadiol (PPT) and protopanaxatriol (PPT), and various sugar moieties attached to the C-3 and C-20 positions (Shi et al. 2010).

Ginsenoside Re (Re, Fig. 1), a main PPT-type ginsenoside, has been identified to possess antioxidant and antioxidant-related properties in various cell types. In chick embryonic cardiomyocytes exposed to hydrogen peroxide and antimycin A, Re attenuates intracellular reactive oxygen species (ROS) levels and diminishes cell death (Xie et al. 2006). Re can protect against the occurrence of oxidative stress due to a depletion of glutathione (GSH) in streptozotocin-induced diabetic rats (Cho et al. 2006). In rats with compound 48/80-induced acute gastric mucosal lesions, Re diminished neutrophil infiltration and enhanced lipid peroxidation in the gastric mucosal tissue (Lee et al. 2014). Re exerts a beneficial effect on neuroinflammatory events in neurodegenerative diseases through phospho-p38, inducible nitric oxide synthase and cyclooxygenase 2 signaling pathways in microglia cells (Lee et al. 2012). The already identified antioxidative properties of Re could be proposed mostly from the experimental models under abnormally derived conditions.

ROS are typically generated as the by-products of mitochondrial respiration or by the actions of oxidases such as NADPH oxidase and xanthine oxidase, and also from the interaction of biological molecules with ionizing radiation (Han and Park 2010). ROS at normal levels mediate many cellular processes, but excessive ROS can disrupt redox homeostasis that subsequently induces oxidative stress (Zuo et al. 2015). Although the intracellular ROS levels are counterbalanced by physiological antioxidants, the aberrantly augmented levels of ROS can lead to diverse disorders comprising myocardial infarction, autoimmune diseases, atherosclerosis, Alzheimer's and Parkinson's diseases and emphysema (Sharma et al. 2015). In some potentially negative aspects, ROS can be thought to be involved in pathological processes more deeply than in normal physiological processes.

Human epidermal keratinocytes, which are always exposed to external stimuli, constantly generate ROS, and sometimes suffer from excessive ROS generated by various stresses, including UV irradiation (Bito and Nishigori 2012). ROS, generated in the skin exposed to UV radiation, attenuate the physiological levels of a number of antioxidants, including GSH, in the epidermis and dermis and thus impair the cellular redox system (Hanada et al. 1997). Since the impairment of redox homeostasis is a major cause of skin photoaging and more serious skin conditions, the defense against oxidative stress caused by UV radiation, particularly the UV-B component, is indispensable, especially for the elderly.

In this work, we demonstrate that Re scavenges the intracellular ROS and NO but enhances the total GSH contents and superoxide dismutase (SOD) activity and that Re downregulates proMMP-2 and -9 under normal conditions.

2. Investigations and results

2.1. Suppression on intracellular ROS levels

HaCaT keratinocytes were subjected to varying concentrations (5, 12 or 30 μM) of Re, and three fluorescent ROS probes, DCFH-DA (Fig. 2A), DHE (Fig. 2B) and DHR (Fig. 2C) were used to quantitate the intracellular ROS. When detected with DCFH-DA, Re could concentration-dependently attenuate the ROS levels (Fig. 2A).

Re at the concentrations of 5, 12 and 30 µM diminished the intracellular ROS levels to 88.5, 78.7 and 72.4% of those of the non-treated cells, respectively (Fig. 2A). As shown in Fig. 2B, when detected with DHE, Re similarly exhibited the attenuating effects on the ROS levels (Fig. 2B). Re at 5, 12 and 30 µM could attenuate the ROS levels to 86.8, 69.8 and 57.6% of those of the non-treated cells, respectively (Fig. 2B). As shown in Fig. 2C, Re also displayed an attenuating effect on the ROS levels when also determined using DHR. Collectively, Re has a suppressive activity on the ROS levels in HaCaT keratinocytes under normal, unstressed conditions, implying its antioxidative property.

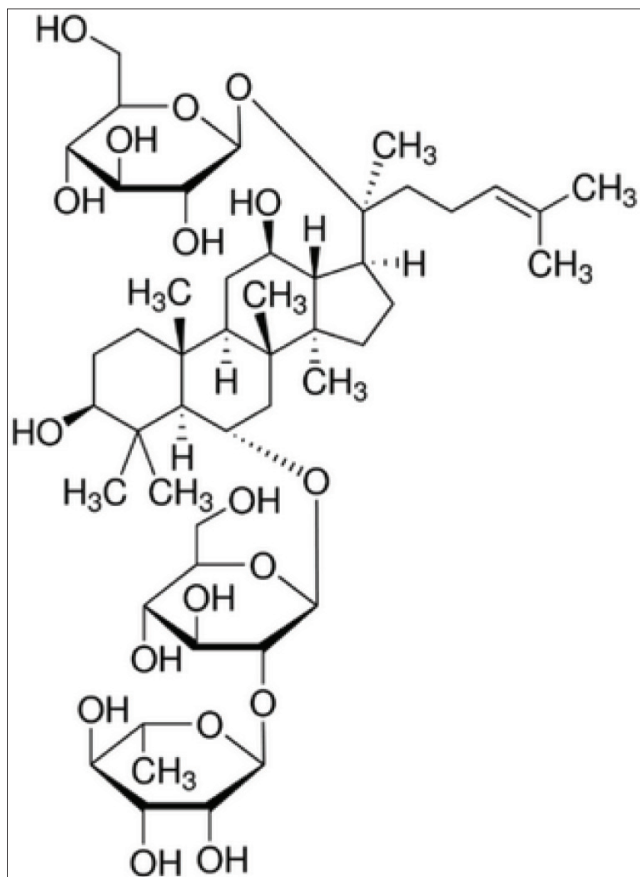

Fig. 1: The chemical structure of ginsenoside Re (Re).

2.2. Suppression on NO levels

Nitric oxide, synthesized in minute amounts by constitutive nitric oxide synthases, participates in normal physiological processes. In contrast, it exhibits pathologic effects when synthesized in excessive amounts by inducible nitric oxide synthases, which respond to pro-inflammatory agents. When the cultured HaCaT keratinocytes were subjected to varying concentrations (5, 12 or 30 µM) of Re, the levels of nitrite, an index of NO, dropped to 91.5. 80.0 and 75.7% of those of the non-treated cells, respectively (Fig. 3). Taken together, Re has a suppressive activity on the NO levels in HaCaT keratinocytes under normal, unstressed conditions.

2.3. Nontoxicity on cellular viability

To test whether Re at concentrations used exhibits cytotoxicities on HaCaT keratinocytes or not, its effect on the cellular viabilities of HaCaT keratinocytes was determined using the MTT assay. Re displayed no cytotoxicities and gave rise to the similar cellular viabilities, compared with the non-treated control (Fig. 4). Collectively, Re, at the used concentrations, is nontoxic to HaCaT keratinocytes.

2.4. Attenuation on proMMP-2 and -9 activities

When HaCaT cells were subjected to Re at 5, 12 and 30 µM, the proMMP-2 gelatinolytic activities in conditioned medium of the

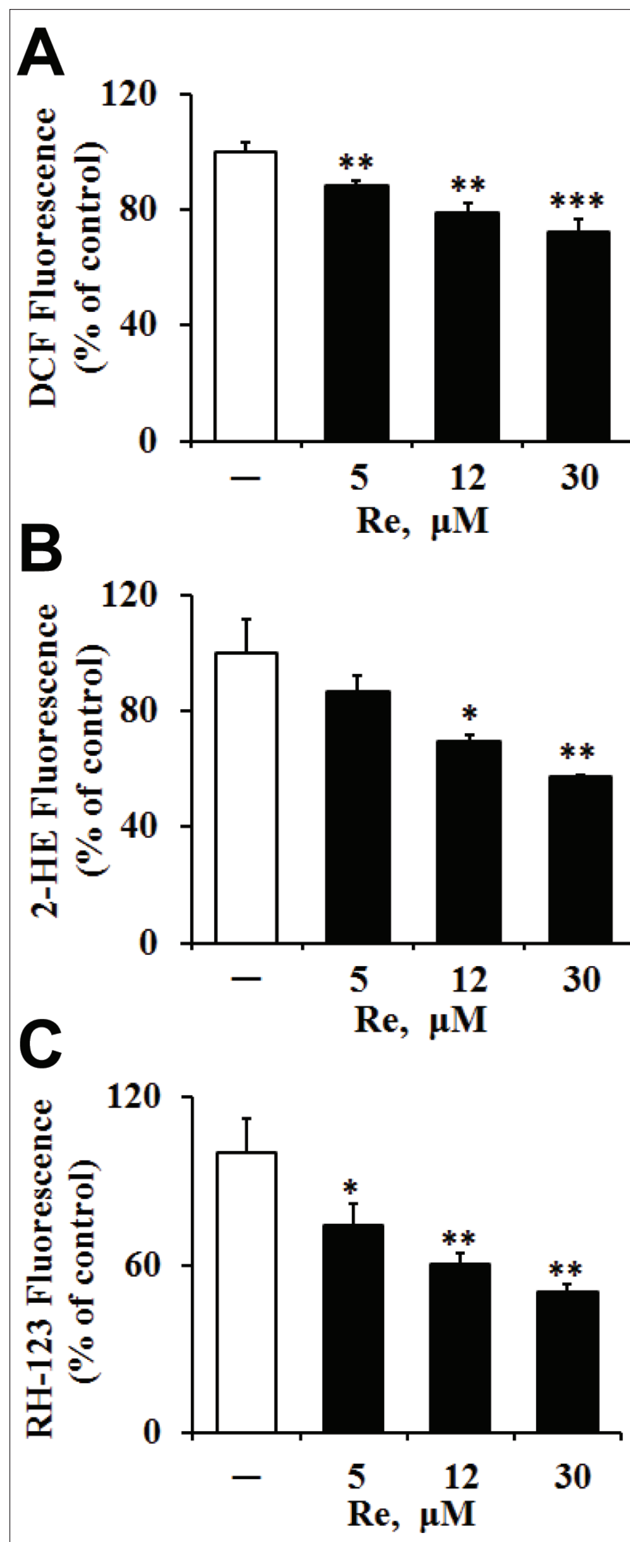

Fig. 2: Suppressive effects of Re on the reactive oxygen species (ROS) levels in HaCaT keratinocytes. The HaCaT cells were incubated in fresh medium for 24 h, and subjected to the indicated concentrations (0, 5, 12 and 30 µM) of Re for 1 h. The ROS level was determined by DCFH-DA (**A**), DHE (**B**) and DHR (**C**) using fluorometry. The ROS level was represented as dichlorofluorescein (DCF, **A**), 2-hydroxyethidium (2-HE, **B**) and rhodamine 123 (RH-123, **C**) fluorescences, arbitrary units expressed as % of control. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ versus the non-treated control.

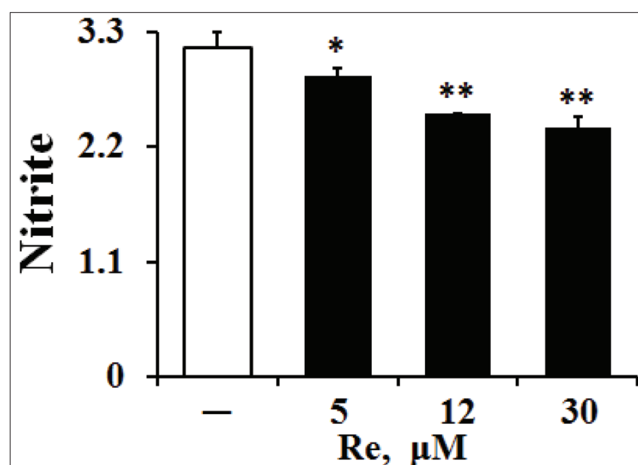

Fig. 3: Suppressive effects of Re on the levels of nitrite, an index of nitric oxide (NO), in HaCaT keratinocytes. The HaCaT cells were incubated in fresh medium for 24 h, and subjected to the indicated concentrations (0, 5, 12 and 30 µM) of Re for 1 h . The nitrite contents in conditioned media were determined by using Griess reagent. The nitrite levels were represented in µM. *, P<0.05; **, P<0.01 versus the non-treated control.

treated cells dropped to 95.0, 77.3 and 46.7%, respectively, of those of the non-treated cells (Fig. 5A). Re at 5, 12 and 30 µM could also attenuate the proMMP-9 gelatinolytic activities to 87.7. 74.3 and 44.0% of those of the non-treated cells, respectively (Fig. 5B). The attenuating effects of Re on both proMMP-2 and -9 activities tend to be occurring depending on its concentrations. These results, obtained from zymographic analyses, suggest that Re is capable of attenuating both proMMP-2 and -9 gelatinolytic activities in HaCaT keratinocytes under normal conditions.

2.5. *Diminishment on proMMP-2 and -9 protein levels*

Since Re was clearly able to attenuate the proMMP-2 and -9 gelatinolytic activities in HaCaT cells (Fig. 5), the effects of Re on proMMP-2 and -9 protein levels in cellular lysates were also determined using western blotting analysis (Fig. 6). As shown in Fig. 6A, Re, at the concentrations of 5, 12 and 30 µM, could diminish the proMMP-2 protein levels in the treated cells to 94.3, 78.0 and 53.3%, respectively, of those of the non-treated cells (Fig. 6A). Similarly, the proMMP-9 protein levels in cellular lysates were attenuated by Re in a concentration-dependent manner (Fig.

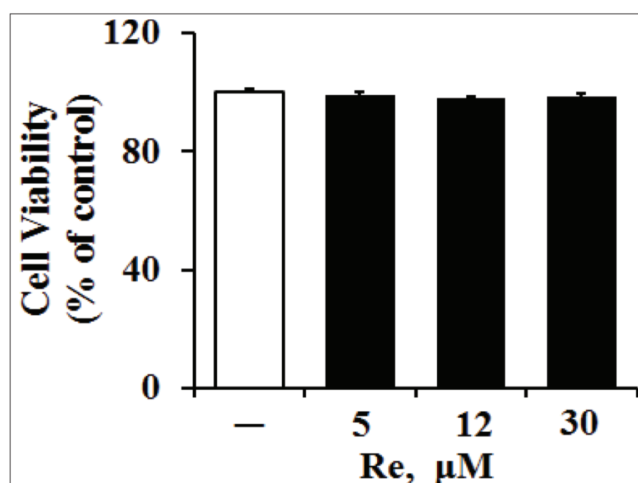

Fig. 4: Effects of Re on the cellular viabilities in HaCaT keratinocytes. The 1.0 x 10^5 HaCaT cells were incubated for further 24 h and pre-treated in the fresh medium with the indicated concentrations (0, 5, 12 and 30 µM) of Re for 1 h. The viable cell numbers, determined using the MTT assay, were expressed as % of control.

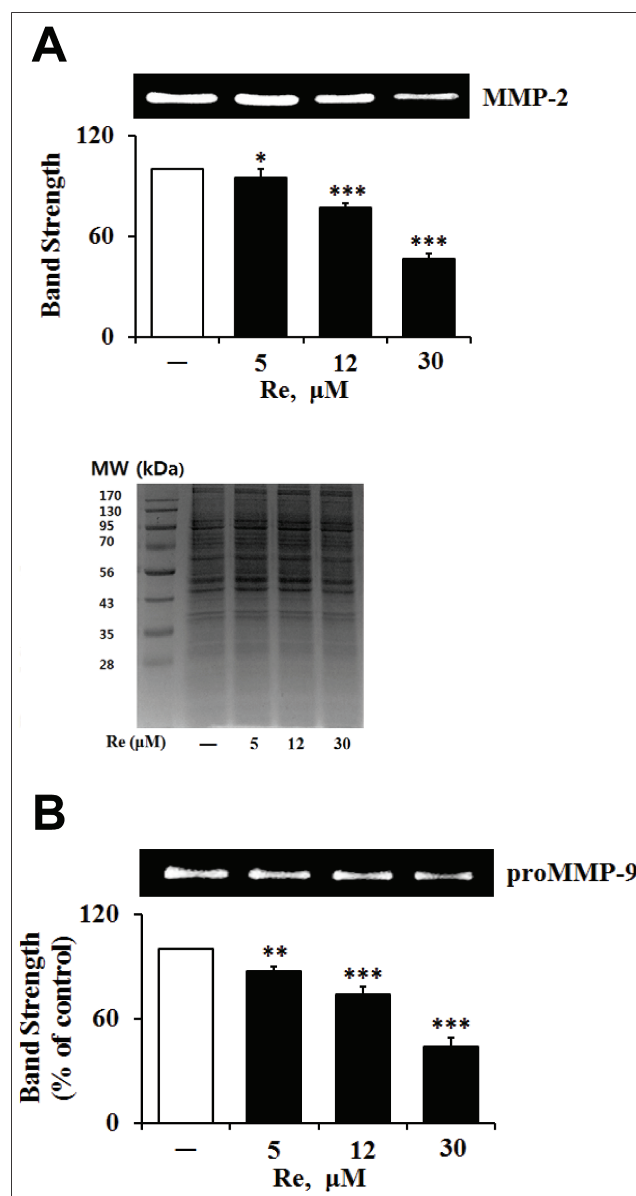

Fig. 5: Suppressive effects of Re on pro-matrix metalloproteinase-2 (proMMP-2, **A**) and -9 (proMMP-9, **B**) gelatinolytic activities in conditioned medium of HaCaT keratinocytes. The HaCaT cells were incubated in fresh medium for 24 h, and subjected to the indicated concentrations (0, 5, 12 and 30 µM) of Re for 1 h, and continued to be incubated for further 24 h. In the lower panel of A, the equal loading of conditioned media was shown by the use of Coomassie blue staining of the identical gel. The relative band strength was determined with densitometry using the ImageJ software which can be downloaded from the NIH website. The gelatinolytic activities of proMMP-2 and -9 in conditioned medium, expressed as % of control, were detected using gelatin zymography. *, P<0.05; **, P<0.01; ***, P<0.001 versus the non-treated control.

6B). Re at 5, 12 and 30 µM could attenuate the proMMP-9 protein levels to 75.0, 57.3 and 39.0%, respectively, of those of the non-treated cells (Fig. 6B). Collectively, Re is able to downregulate the proMMP-2 and -9 production in HaCaT keratinocytes, which corresponds to the attenuation by Re of proMMP-2 and -9 gelatinolytic activities in conditioned medium.

2.6. *Upregulation of total GSH and SOD*

Re at 5, 12 and 30 µM enhanced the total GSH contents in the treated cells by 1.4-, 1.9- and 2.5-fold, respectively, compared to those of the non-treated cells that did not receive the treatment of Re (Fig, 7A). This finding implies that the GSH-enhancing effects of Re might be related to maintaining the cellular redox homeostasis in HaCaT keratinocytes.

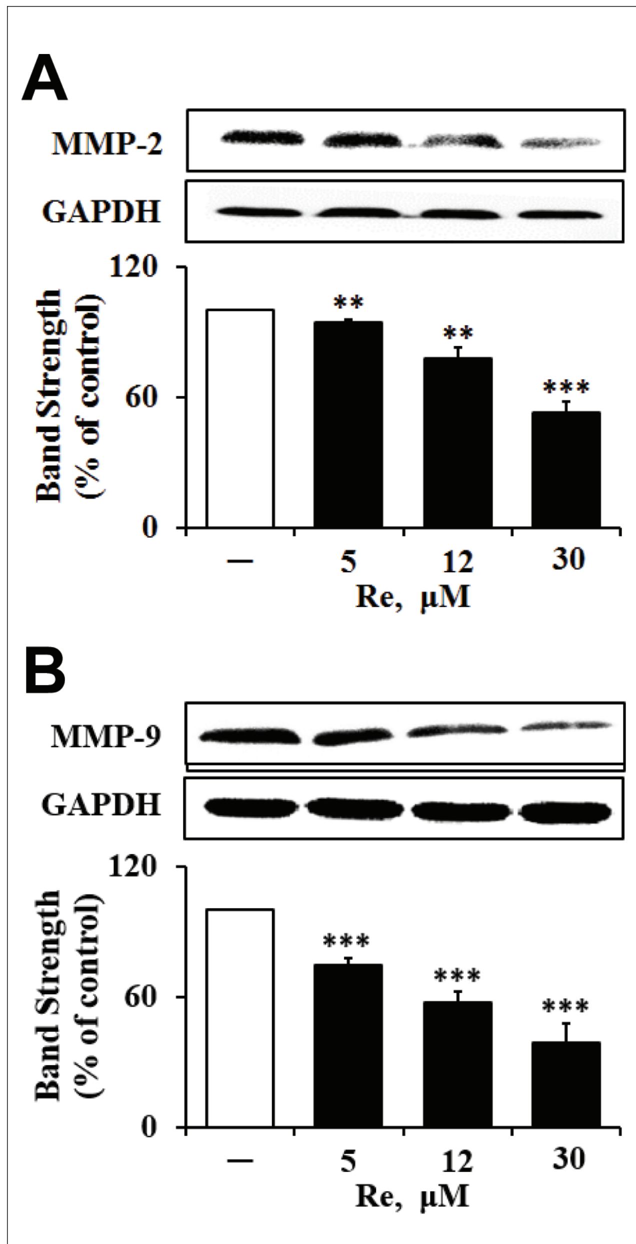

Fig. 6: Suppressive effects of Re on pro-matrix metalloproteinase-2 (proMMP-2, **A**) and -9 (**B**) protein levels in cellular lysates of HaCaT keratinocytes. The HaCaT cells were subjected to the indicated concentrations (0, 5, 12 and 30 µM) of Re for 1 h. The proMMP-2 and -9 proteins, expressed as % of control, were determined using western blotting analysis with anti-MMP-2 and -9 antibodies. GAPDH was used as a protein loading control. The relative band strength, expressed as % of control, was determined with densitometry using the ImageJ software which can be downloaded from the NIH website. **, P<0.01; ***, P<0.001 versus the non-treated control.

SOD, which catalyzes the conversion of superoxide anion into hydrogen peroxide and molecular oxygen, is one of the crucial antioxidant enzymes, since the superoxide anion is thought as one of more toxic ROS species. Since Re was shown to have a GSH-enhancing activity in Fig. 7A, its effect on total SOD activity could also be of interest. As shown in Fig. 7B, the total SOD activities in the cells subjected to Re at 5.0, 12.0 and 30.0 µM were enhanced to 1.5-, 1.7- and 2.0-fold, respectively, compared to those of the non-treated keratinocytes without the exposure to Re (Fig. 7B). In brief, Re is capable of enhancing SOD activity in HaCaT keratinocytes under normal conditions. The SOD-enhancing activity of Re, together with its GSH-enhancing activity, may be a mechanism underlying its oxidative property.

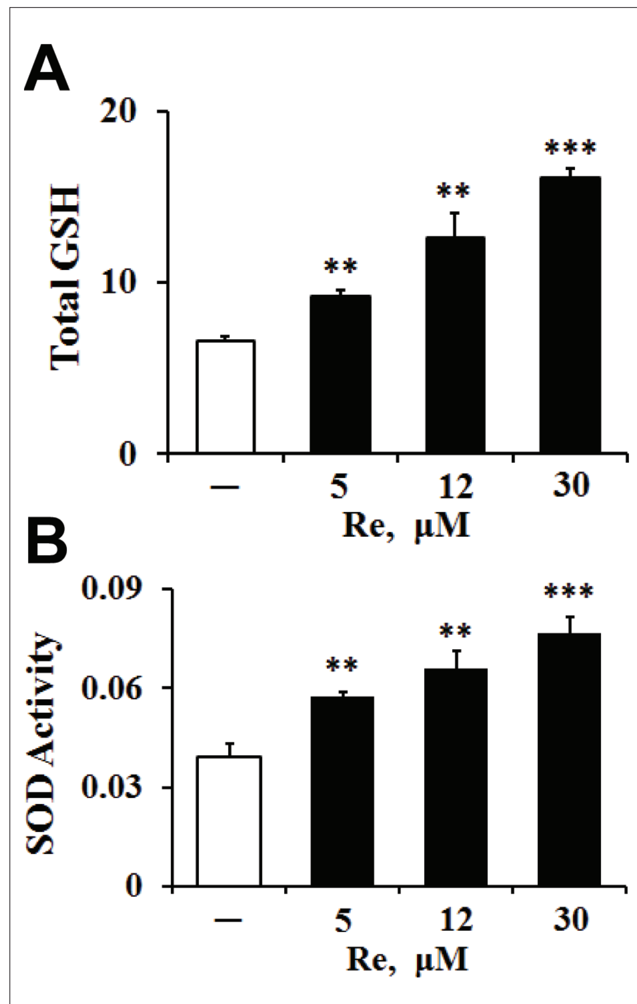

Fig. 7: Enhancing effects of Re on total glutathione (GSH, **A**) and superoxide dismutase (SOD, **B**) activity levels in cellular lysates of HaCaT keratinocytes. The HaCaT cells were subjected to the indicated concentrations (0, 5, 12 and 30 µM) of Re for 1 h. In **A**, total GSH content, expressed as µg/mg protein, was determined with enzymatic recycling assay using GR. In **B**, total SOD activity, expressed as Δ_{550}/min/mg protein, was measured using a spectrophotometric assay.

3. Discussion

Living cells frequently crash into adverse environments one of which might be an oxidative stress state. Appropriate maintenance of redox homeostasis is more important to some kinds of skin cells, such as epidermal keratinocytes and dermal fibroblasts, in which ROS are repeatedly produced due to their exposure to sunlight and oxygen. For example, exposure to the sun's UV radiation induces the formation of ROS which can produce cytotoxic effects on cells by oxidizing macromolecules and genotoxic effects by inducing base lesions in DNA (Cadet et al. 1997). ROS are able to sequentially induce the degradation of collagen and elastin, the main components of extracellular matrix, through enhancing the activities of matrix metalloproteinases, which leads to the skin's aging. To cope with repeatedly generating ROS, aerobic cells are evolutionarily equipped with complex systems of multiple types of antioxidants, including antioxidant enzymes such as SOD and catalase and GSH. Decreased levels of SOD, catalase or GSH can cause an accumulation of ROS and contribute to the enhanced cellular redox state which induces cellular damage by oxidation of nucleic acids, proteins and lipids (Ryu et al. 2011). Since atopic dermatitis patients are more prone to damage caused by ROS or oxidants, which were evident from an increase of malondialdehyde and a decrease of enzymatic and non-enzymatic antioxidants, antioxidants were suggested to be beneficial in the treatment (Sivaranjani et al. 2013). Accordingly, normal redox

homeostasis in skin cells is needed to preserve healthy skin and to prevent several serious skin diseases, especially at older ages.

Various components and mixtures have been identified to be involved in the modulation of the ROS levels in cultured keratinocytes and fibroblasts. Many of them display their modulating roles in redox homeostasis via regulating GSH content or GSH/GSSG ratio and other antioxidant components. Aucubin, a common iridoid glucoside with various pharmacological activities, such as hepatoprotective, collagen synthesizing and anti-inflammatory effects, plays a crucial role in cellular defense against UV-B-induced photoaging through inhibiting ROS formation and malondialdehyde levels but enhancing GSH levels (Ho et al. 2005). Piceatannol, a phenolic compound occurring naturally in grapes and red wine, exhibits an anti-melanogenic action through a combination of its antioxidative property, suppressive effect on ROS generation, and enhancing effect on GSH/GSSG ratio (Yokozawa and Kim 2007). Two heptapeptides, purified from seaweed pipefish, possess a hydroxyl radical scavenging activity, and suppress the hydrogen peroxide-induced ROS production and DNA damage in human dermal fibroblast cells via increasing the expression levels of SOD, GSH and catalase through blocking the NF-κB activation, leading to the reduction of oxidative stress-mediated damage (Ryu et al. 2011). An extract of the fern *Polypodium leucotomos*, named fernblock, inhibits generation of ROS induced by UV radiation, including superoxide anion, prevents damage to the DNA, inhibits UV-induced AP1 and NF-κB, and protects endogenous skin natural antioxidant systems, such as catalase and GSH (Gonzalez et al. 2011). An extract of tamarind seed coat, widely used in various traditional medicine and food products, attenuates intracellular ROS in the absence and presence of hydrogen peroxide by increasing GSH level (Nakchat et al. 2014). In the absence of hydrogen peroxide, it enhances SOD and catalase activity but does not affect glutathione peroxidase. Meanwhile, it increases the expression of SOD and GSH peroxidase in hydrogen peroxide-treated human foreskin fibroblasts (Nakchat et al. 2014).

Exogenously added modified GSH or GSH precursor could be more effective, since free GSH is not therapeutically effective due to its unfavorable pharmacokinetic properties. UV-induced toxicity, characterized by marked oxidative stress accompanied by the depletion of key cellular antioxidants, particularly GSH, is counteracted by replenishing cellular GSH. Thus, *S*-acyl-glutathione derivatives, rather than GSH, increase intracellular levels of reduced GSH in primary skin fibroblasts, protects against UV-induced ROS production and UV-B/C-mediated lipid peroxidation and caspase-3 activation (Wright et al. 2013). *N*-Acetyl-l-cysteine protects human fibroblasts directly by scavenging ROS induced by UV-A and visible radiation, and indirectly by donating l-cysteine for GSH biosynthesis (Morley et al. 2003). These results might imply that GSH-enhancing agents or GSH derivatives are better choices than GSH itself to enhance intracellular antioxidant levels for the purpose of maintaining normal redox homeostasis in skin.

The composition of ginsenosides in the ginseng plants are affected by various factors such as species, age, part of the plant, cultivation method, harvesting season and preservation method (Schlag and McIntosh 2006). Re, used as a model ginsenoside in this work, was confirmed to be one of main ginsenosides in the commercial ginseng products such as the ginseng capsules (Uhr et al. 2014). At present, the pharmacological effects of ginseng are generally attributed to ginsenosides in most cases. Considering that the dammarane parts of ginsenosides are possibly a better candidate to be responsible for ginseng's pharmacological effects than sugar moieties, many ginsenosides would be assumed to exhibit their pharmacological effects in a few common mechanisms. Currently, those kinds of action mechanisms have not been clearly suggested for dammarane ginsenosides. One suggestion is that ginsenosides can be used as natural resources to be developed into new modalities to replace steroids in the current regimen to lessen undesirable side effects, based on their actions as partial agonists to multiple steroidal receptors (Leung and Wong 2010). Although other possibility can be assumed to center on the antioxidative activities, the related common mechanism(s) have not been correctly set up. In this work, we demonstrate that Re is capable of enhancing the total GSH and SOD activity levels in HaCaT keratinocytes under normal conditions. The elevated GSH and SOD levels subsequently support the downregulating effects of Re on the ROS levels and MMP-2 and -9, implying the indirect antioxidative activities of Re through upregulating the total GSH and SOD levels. The results also propose that HaCaT keratinocytes having the GSH and SOD levels elevated by Re can be advantageous to preserve redox homeostasis under oxidative stress conditions, including UV radiation and other oxidants. If these findings apply to other ginsenosides, it would be very interesting to become a plausible common mechanism. However, further approaches are required to confirm the possibility.

In this work, we demonstrate that Re is capable of downregulating proMMP-2 and -9 at both activity and protein level. MMPs, a complex family of zinc-containing proteinases, can degrade the components of extracellular matrix forming skin dermal connective tissue (Curran and Murray 1999). Some MMPs are produced and released by cells in the precursor forms (proMMPs), and their activation is controlled by a cascade of steps involving other MMPs and the plasmin system (Dzwonek et al. 2004). Excessive ROS under oxidative stress in conjunction with the resulting inflammation upregulate the over-expression of MMPs, which in turn causes the degradation of extracellular matrix, finally leading to coarse wrinkling, dryness, and laxity of the skin (Feng et al. 2014). UV-B radiation was previously found to significantly enhance proMMP-2 and -9 mRNAs and gelatinolytic activities in HaCaT keratinocytes (Kim et al. 2013). ROS is considered as a major factor to initiate the upregulation of MMPs in keratinocytes and fibroblasts via the activation of receptor proteins on the cell membrane of those cells, and to degrade fiber components in dermis, leading to wrinkle formation (Inui et al. 2008). Together with the previous findings, the current results imply that Re downregulates proMMP-2 and -9 through the diminishment of ROS in HaCaT keratinocytes under normal conditions.

In conclusion, Re downregulates the ROS and NO levels and MMP-2 and -9, and upregulates the total GSH contents and SOD activity in HaCaT keratinocytes under normal conditions. These findings made us to propose that Re possesses an antioxidative activity occurring through the upregulation of antioxidant components, including total GSH and SOD, even under normal conditions and that it can has preventive properties on the occurrence of oxidative stress in HaCaT keratinocytes. They also suggest that Re and other ginsenosides have similar capabilities in the absence or presence of stress.

4. Experimental

4.1. Materials and reagents

Ginsenoside Re (Re, purity≥98%) was purchased from Ambo Institute (Seoul, Korea). Griess reagent, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), dihydroethidium (DHE), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dihydrorhodamine 123 (DHR), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione reductase (GR), reduced glutathione (GSH), NADPH, cytochrome c, xanthine, and xanthine oxidase were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin were from HyClone Laboratories Inc. (Logan, UT, USA). Cell lysis buffer was obtained from Promega Korea (Seoul, Korea). All other chemicals used were of the highest grade commercially available.

4.2 Cell culture

An immortalized HaCaT keratinocyte cell line (ATCC, Manassas, VA, USA) was cultured in DMEM containing 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. Prior to the treatments, 1 x 10^5 HaCaT keratinocytes were seeded on 24-well plates and grown overnight, washed twice with 1 ml phosphate-buffered saline (PBS), and replaced with 1 ml FBS-free medium. After the treatments, the cells were incubated under the same culture conditions.

4.3. Cellular lysate preparation

After adherent cells were washed twice with PBS and stored on ice for 5 min, the cells were harvested by scraping off the bottom of the dish with a cell scraper and by centrifugation at 15,000 rpm for 10 min. The cell pellets were resuspended in cell lysis buffer [25 mM Tris-phosphate (pH 7.8), 2 mM 1,2-diaminocyclohexane-N,N,Nv,Nv-tetraactic acid, 2 mM dithiothreitol, 10% glycerol, 1% Triton X-100] and stored for 30 min on ice. Cellular lysates or supernatant fluids were taken after centrifugation at 15,000 rpm for 15 min.

Protein contents in cellular lysates were determined according to the Bradford assay (Bradford 1976).

4.4. Quantitation of intracellular ROS

As previously described (Royall and Ischiropoulos 1993), a redox-sensitive fluorescent probe DCFH-DA, which generates the fluorescent 2',7'-dichlorofluorescein (DCF; $\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 530 nm) upon enzymatic reduction and subsequent oxidation by ROS, was used to fluorometrically determine intracellular ROS. After the treatment with Re and/or 20 µM DCFH-DA for 30 min at 37 °C, the cells were twice washed with 1 ml FBS-free medium, and resuspended in 1 ml FBS-free medium. The ROS levels were quantitated by Multi-Mode Microplate Reader (Synergy™ Mx, BioTek Instruments, Winooki, VT, USA). DHE, chiefly used to evaluate intracellular production of superoxide anion in intact cells was utilized in a similar manner. It reacts with superoxide anion to generate 2-hydroxyethidium (2-HE; $\lambda_{excitation}$ = 500 nm, $\lambda_{emission}$ = 580 nm), a final product. DHR, which is a fluorescent mitochondrial dye to react with superoxide, peroxide, peroxynitrite and so on to generate rhodamine 123 ($\lambda_{excitation}$ = 500 nm, $\lambda_{emission}$ = 535 nm), was used according to a similar experimental protocol.

4.5. Quantitation of nitrite

Accumulated nitrite (NO_2^-) in culture supernatants was quantitated using a spectrophotometric assay based upon the Griess reaction (Sherman et al. 1993). An equal volume of Griess reagent (1% sulfanilamide - 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid) was incubated with conditioned medium for 10 min at room temperature and absorbance at 550 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). A calibration curve was constructed using known concentrations (0 – 160 µM) of sodium nitrite (Sigma-Aldrich, St Louis, MO, USA).

4.6. Cell viability assay

The cell viabilities of HaCaT keratinocytes in the presence of Re were determined by the MTT assay used to assess metabolic activity (Freshney 1994). Cells were subjected to Re for 30 min. The cells, after removing the medium, were treated with 5 µg/ml MTT in medium for 4 h. The cells were then lysed with dimethyl sulfoxide, and the amount of formazan, produced from the reduction of MTT by the mitochondria of living cells, was quantified by the absorbance at 540 nm.

4.7. Gelatin zymography

As previously described (Kleiner and Stetler-Stevenson 1994), the proMMP-2 and -9 gelatinolytic activities in conditioned medium were determined. The cells in 1 ml FBS-free medium were treated with Re for 1 h. The conditioned medium, taken from the HaCaT keratinocytes culture incubated for 24 h at 37 °C, was separated on 10% (w/v) SDS-PAGE gel impregnated with 1 mg/ml gelatin under non-reducing condition. The proteins in the gel were renatured by shaking with 2.5% Triton X-100 at room temperature for 30 min, which was repeated two times, and incubated in the incubation buffer (50 mM Tris buffer, pH 7.8, 5 mM $CaCl_2$, 0.15 M NaCl, 1% Triton X-100) for 24 h. After the gel was stained with 0.1% Coomassie Brilliant Blue R-250, the gelatin-degrading enzyme activities were convinced as clear zones against a blue background. proMMP-2 and -9 activity bands were identified in accordance with their molecular masses (72 kDa, 92 kDa), which were estimated by molecular mass markers.

4.8. Western blotting analysis

In order to detect proMMP-2 and -9 proteins in cellular lysate, western blotting analyses were performed using anti-MMP-2 (ALX-210-753, Enzo Life Sciences, Farmingdale, NY, USA) and anti-MMP-9 (3852S, Cell Signaling Technology, Danvers, MA, USA) antibodies as primary antibodies. GAPDH, as an internal standard, in cellular lysate was detected using anti-GAPDH antibody (LF-PA0212, AbFrontier, Seoul, Korea). Cellular lysates were separated on 10% (w/v) SDS-PAGE and electrotransferred to PVDF transfer membrane. The membrane was blocked with blocking buffer (2% bovine serum albumin in 1x TBS-Tween 20), probed with primary antibody overnight at 4 °C, incubated with secondary antibody (goat anti-rabbit IgG-pAb-HRP-conjugate; ADI-SAB-300, Enzo Life Sciences, Farmingdale, NY, USA) for 1 h at room temperature, and developed using an enhanced West-save up ™ (AbFrontier, Seoul, Korea).

4.9. Detection of total GSH and SOD activity

As previously described (Nakagawa et al. 1990), total GSH contents in cellular lysates were detected using an enzymatic recycling assay based on GR. The reaction mixture (200 µl), containing 175 mM KH_2PO_4, 6.3 mM EDTA, 0.21 mM NADPH, 0.6 mM DTNB, 0.5 units/ml GR, and cellular lysate, was incubated at 25 °C. A change in absorbance at 412 nm was monitored using a microplate reader. Total GSH was reported as µg/mg protein.

As previously described (Lee et al. 2002), total SOD activity in cellular lysates was spectrophotometrically determined as the reduction of cytochrome c with xanthine/xanthine oxidase system. The reaction mixture (200 µL) contained 50 mM phosphate buffer (pH 7.4), 0.01 units/ml xanthine oxidase, 0.1 mM EDTA, 1 µM catalase, 0.05 mM xanthine, 20 µM cytochrome c and cellular lysate. A change in absorbance was monitored at 550 nm.

4.10. Statistical analysis

The results were represented as mean ± SD. Differences between experimental groups were analyzed using one-way ANOVA followed by post hoc Tukey HSD test for multiple comparisons. A *P* value less than 0.05 was considered statistically significant.

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