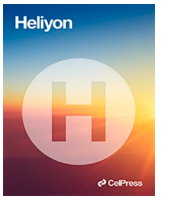
Heliyon 10 (2024) e27688

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Ginsenoside Re protects rhinovirus-induced disruption of tight junction through inhibition of ROS-mediated phosphatases inactivation in human nasal epithelial cells

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ARTICLE INFO

Keywords:
Ginsenoside Re
Human rhinovirus
ROS
Human nasal epithelial cells
Tight junction proteins

ABSTRACT

Maintaining tight junction integrity significantly contributes to epithelial barrier function. If the barrier function is destroyed, the permeability of the cells increases, and the movement of the pathogens is promoted, thereby further increasing the susceptibility to secondary infection. Ginsenoside components have multiple biological activities, including antiviral effects. In this study, we examined the protective effects of ginsenoside Re against rhinovirus-induced tight junction disruption in primary human nasal epithelial cells (HNE). Incubation with human rhinovirus resulted in marked disruption of tight junction proteins (ZO-1, E-cadherin, claudin-1, and occludin) in human nasal epithelial cells. Rhinovirus-induced disruption of tight junction proteins was strongly inhibited by the treatment of cells with ginsenoside Re. Indeed, significant amounts of reactive oxygen species (ROS) have been detected in human nasal epithelial cells co-incubated with rhinovirus. Moreover, rhinovirus-induced ROS generation was markedly reduced by the ginsenoside Re. However, ginsenosides Rb1 and Rc did not inhibit tight junction disruption or ROS generation in nasal epithelial cells following incubation with rhinovirus. Furthermore, incubation with rhinovirus resulted in a marked decrease in protein phosphatase activity and an increase in protein tyrosine phosphorylation levels in nasal epithelial cells. Treatment of cells with ginsenoside Re inhibited rhinovirus-induced inactivation of phosphatases and phosphorylation of tyrosine. Our results identified ginsenoside Re as an effective compound that prevented rhinovirus-induced tight junction disruption in human nasal epithelial cells.

1. Introduction

Panax ginseng C.A. Meyer has many beneficial functions, including anti-cancer, anti-oxidant, anti-aging, and immunostimulatory properties. While Korean red ginseng (KRG) is made by repeated steaming and drying of Panax ginseng, the same components undergo chemical transformation and fortification [1]. Anti-stress [2], anti-oxidant [3], anti-microbial [4], and anti-viral properties [5] of KRG have been established in previous studies. Red ginseng acidic polysaccharides and ginsenosides, which are components of KRG, are known to improve immune system function [6]. Moreover, the antiviral activities of ginseng against the influenza virus [7], norovirus [8], and HBV [9] have recently been reported.

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https://doi.org/10.1016/j.heliyon.2024.e27688
Received 24 April 2023; Received in revised form 4 March 2024; Accepted 5 March 2024
Available online 7 March 2024

Ginsenosides are the major active components of ginseng and exhibit anti-inflammatory effects [10]. These components can be divided into three major groups: protopanaxadiol, protopanaxatriol, and oleanolic acid derivatives [11]. Ginsenoside Re is a major active protopanaxatriol-type compound present in the root and stem leaves of ginseng. Protopanaxatriol-type ginsenoside Re has been shown to undergo transformation into Rg1, F1, and aglycon ginsenoside-PPT (PPT) [12]. Ginsenoside Re exhibits not only neuroprotective activities but also antioxidant effects, and also demonstrates immunomodulatory therapeutic properties [13,14] and anti-inflammatory effects [15]. A previous study showed that Ginsenoside Re exerts anti-inflammatory effects by inhibiting NO and NF-κB signaling in LPS-activated N9 microglial cells [16].

Human rhinovirus (HRV) is one of the most important etiological agents of the common cold [17]. Although HRV-induced upper respiratory illnesses are usually mild and self-limiting, there is increasing evidence linking HRV infection to more serious medical complications, including asthma exacerbation [18]. Previous studies have reported that rhinovirus disrupts barrier function by altering tight junction components in epithelial cells [19,20]. Moreover, there is recent evidence that the generation of intracellular reactive oxygen species (ROS) plays an important role in tight junction disruption by rhinoviruses in nasal epithelial cells [21].

Reactive oxygen species are cytotoxic, but they may also act as secondary messengers in intracellular signaling, and excessive ROS can induce stimulation of pro-inflammatory genes, tissue damage, and cell differentiation [22,23]. ROS induce the disruption of tight junctions in a variety of epithelial tissues [24,25]. In addition, oxidants may increase protein tyrosine phosphorylation by inhibiting protein tyrosine phosphatase (PTPase), thereby increasing barrier disruption [26].

Previously, we reported that phosphatase inhibition by ROS was associated with tight junction disruption by RV in nasal epithelial cells [21]. Based on previous research, we investigated whether ginsenoside Re prevents ROS production and prevents protein tyrosine phosphatase (PTPs) inactivation to protect the epithelial barrier function from disruption by RV.

Several studies have reported the anti-viral effects of other ginsenosides but not Re. Therefore, we examined the effect of ginsenoside Re on the HRV 16-induced increase in tight junction disruption in human nasal epithelial cells and provided insight into its molecular mechanism. This study provides further evidence supporting ginsenoside Re as a potential anti-inflammatory drug and facilitates the development of new therapeutic strategies.

2. Material and methods

2.1. Reagents and antibodies

Ginsenoside Re, Rb1, and Rc were purchased from the Ambo Institute (Daejeon, Korea). Diphenyleneiodonium (DPI) was obtained from EMD Biosciences (Darmstadt, Germany). H_2DCFDA was purchased from Molecular Probes (Eugene, OR). Antibody against occludin was purchased from Abcam (Cambridge, UK). Antibodies against ZO-1, claudin, E-cadherin, phosphotyrosine proteins and β-actin (Rabbit) were purchased from Cell Signaling Technology (Beverly, MA, USA). Unless otherwise specified, reagents were purchased from Merck/Sigma-Aldrich (Darmstadt, Germany).

2.2. Primary cell culture

The present study was approved by the Institutional Review Board of Gachon University Gil Medical Center (No. GCIRB2014-362). Human nasal mucosal tissues were collected from patients who underwent inferior turbinectomy. The cell culture method used in the experiment was described in a previous paper published by the author of this study [21].

2.3. Cell viability assay

To analysis cell viability, HNE cells were incubated with ginsenosides Re, Rb1, and Rc for 2 h at 37 °C. After incubation, cell viability was measured using an EZ-Cytox cell viability assay kit (Daeil Lab, Seoul, Korea) according to the manufacturer's instructions.

2.4. HRV 16 infection and ginsenoside treatment

Primary HNE cells at passage two were used for the experiments. HRV16 stocks were amplified and purified based on the published protocol [21]. HNE cells were incubated with HRV16 (5×10^5 $TCID_{50}$/mL) for 4 h at 33 °C. After incubation, the suspension was removed and the cells were washed with PBS, which was replaced by fresh medium at the end of the washing step. HRV16-infected HNE cells were cultured for 24, 48, and 72 h in the presence or absence of ginsenosides Re, Rb1, and Rc.

2.5. Immunoblotting

HNE cells infected with or without HRV16, were incubated in the absence or presence of ginsenosides for 72 h. After incubation, the cells were lysed with RIPA buffer on ice for 10 min as previously described [21]. Equal amounts of protein were subjected to SDS-PAGE and subsequently electrotransferred onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline Tween 20 at room temperature for 1 h, followed by incubation with proper primary antibodies against ZO-1, occludin, claudin, E-cadherin, phospho-tyrosine protein, or β-actin at 4 °C overnight. The membranes were then incubated with HRP-conjugated anti-rabbit or mouse antibody at room temperature for 1 h. Immunoreactivity was detected using an ECL detection system (Bio--Rad, CA, USA).

2.6. Measurement of TEER

To analysis transepithelial electrical resistance by HRV16, HNE cells were cultured in the inner chambers of Transwell inserts (0.4 μm) (Corning Life Sciences, MA, USA). After incubation, HNE cells infected with or without HRV16, were incubated in the absence or presence of ginsenosides for 72 h. TEER of HNE cells was measured using an EVOM ohm-voltameter (World Precision Instruments, FL, USA). For calculation, the resistance value of the blank was subtracted from that of covered with cells. The values were expressed in standard units of ohms per cm^2.

2.7. Measurement of ROS generation

Intracellular ROS production in HNE cells was measured using the green fluorescence probe, H_2DCF-DA. In brief, HNE cells were cultured for 24 h with or without HRV 16 in a CO2 incubator at 33 °C. After incubation, HNE cells were stained at 37 °C for 30 min with H_2DCF-DA (1 μM), which is rapidly oxidized to highly fluorescent DCF in the presence of intracellular H_2O_2. Cells were washed twice with PBS, and DCF fluorescence was measured by FACScan (Becton Dickinson, CA, USA) and fluorescence microscopy (Zeiss, Oberkochen, Germany). To flow cytometric analysis, at least 10,000 gated events were analyzed for each sample. Fluorescence microscopy was analyzed using a magnification of × 200. To examine the effect of ginsenoside components on HRV 16-induced ROS production in HNE cells, cells were treated with ginsenosides Re, Rb1, and Rc.

2.8. Measurement of phosphatase activity

To elucidate the tyrosine phosphatase activity of HNE cells induced by HRV 16, HNE cells were incubated with HRV 16 at 33 °C for 48 h. To examine the effect of ginsenoside Re on the reduction in phosphatase activity induced by HRV 16 in HNE cells, cells were treated with ginsenoside Re. After incubation, phosphatase activity was measured using a non-Radioactive Tyrosine Phosphatase Assay System kit (Promega, Madison, WI) according to the manufacturer's instructions.

2.9. Statistical analysis

All reactions were expressed as the mean ± standard deviation (SD) from 3 to 5 independent experiments. Statistical analysis was performed using Student's *t*-test. In all analyses, $P < 0.05$ was considered statistically significant.

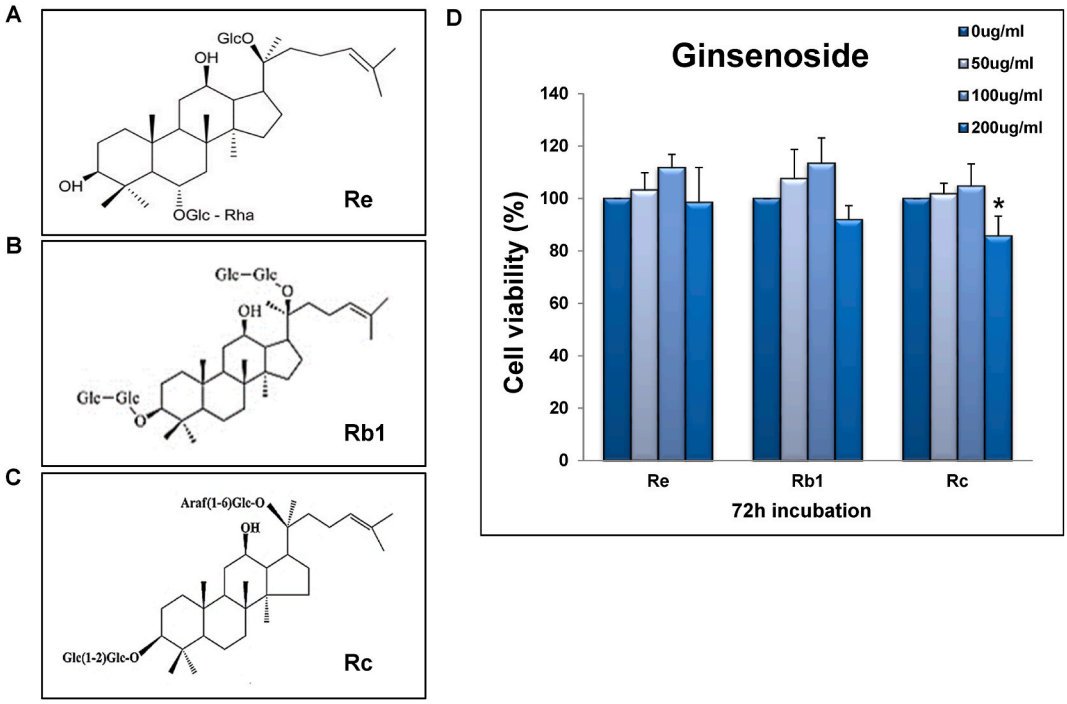

Fig. 1. Effect of ginsenoside components on the viability of human nasal epithelial (HNE) cells. (A)–(C) Chemical structure of ginsenoside Re, Rb1, and Rc. (D) Human nasal epithelial cells were incubated in the presence or absence of ginsenoside Re, Rb1, and Rc (50 μg/mL to 200 μg/mL) for 72 h. Bar graph indicates the effect of ginsenoside Re, Rb1, and Rc on HNE cell viability. The values were expressed as mean ± SEM of three independent experiments. *$P < 0.005$ vs. untreated HNE cells.

3. Results

3.1. Effect of ginsenoside components on viability of HNE cells

Ginsenosides are composed generally of the protopanaxadiol (PPD) and protopanaxatriol (PPT) groups. The two groups are distinguished according to the position of the sugar moities attached to the dammarane-type triterpine. As shown in Fig. 1A–C, ginsenoside Re belongs to the PPT group, while Rb1 and Rc belong to the PPD group. The cytotoxicity of ginsenoside components (Re, Rb1, and Rc) in HNE cells was determined by cell viability analysis. As shown in Fig. 1D, ginsenosides Re, Rb1, and Rc had no cytotoxic effects at a dose of 100 μg/mL. However, ginsenoside Rc exhibited cytotoxicity at a higher concentration (200 μg/mL).

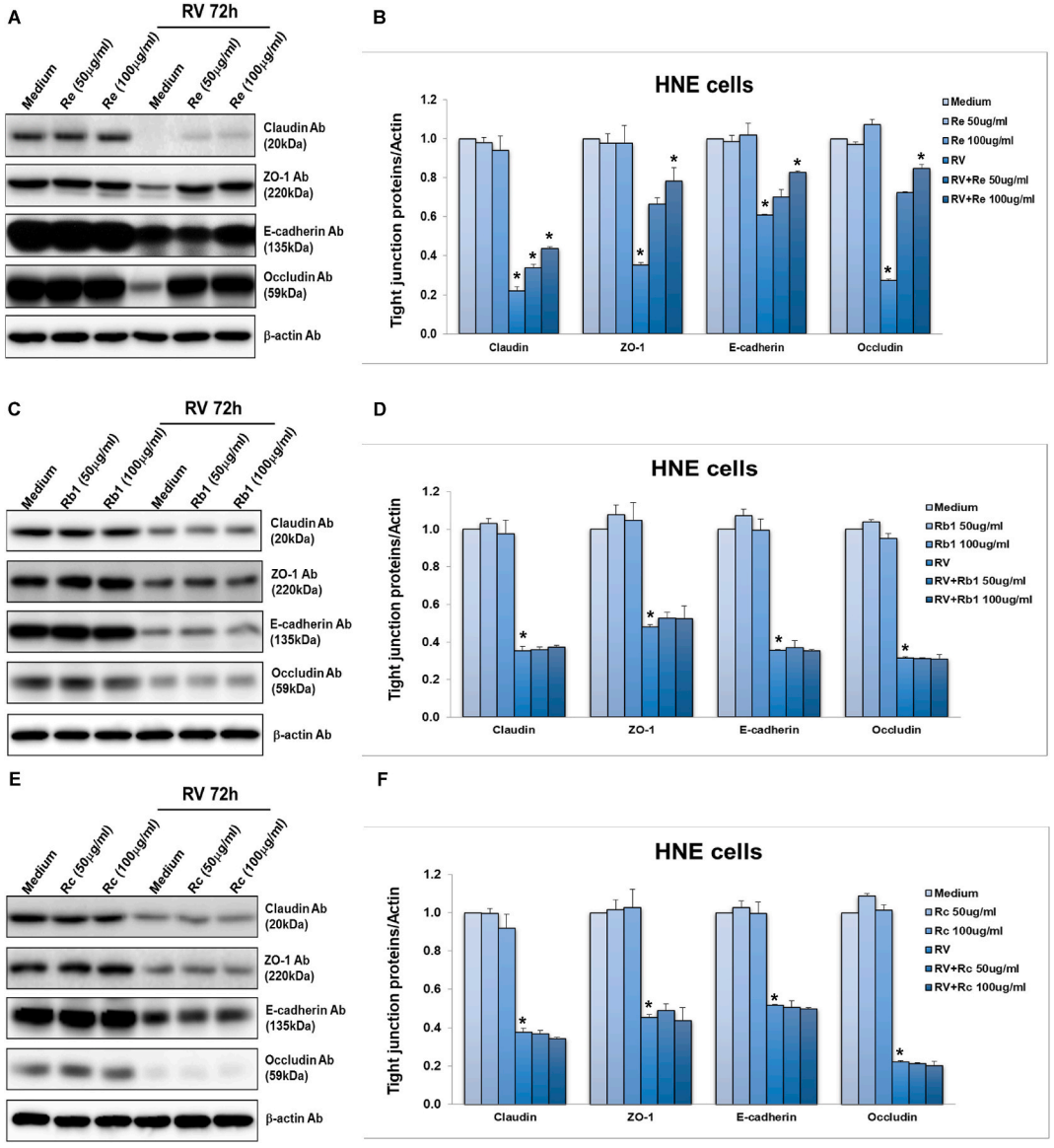

Fig. 2. Effect of ginsenoside components on tight junction disruption of HNE cells induced by human rhinovirus 16 (HRV 16). HNE cells were incubated for 72 h at 37 °C with or without HRV 16 in the absence or presence of ginsenoside Re, Rb1, and Rc (50 μg/mL and 100 μg/mL). After incubation, proteins in whole-cell lysates were subjected to SDS-PAGE and subsequently western blotted with anti-occludin, anti-ZO-1, anti-claudin, and anti-E-cadherin antibodies. β-Actin was used as a loading control. Figures are representative of three independent experiments, each showing similar results.

3.2. Effects of ginsenoside Re on tight junction disruption

To examine the potential effect of ginsenoside components in tight junction disruption induced by HRV 16, we treated HNE cells with ginsenosides Re, Rb1, and Rc. As shown in Fig. 2A and B the treatment of ginsenoside Re prevented HRV 16-induced disruption of tight junction proteins in HNE cells. In particular, at 100 μg/mL, ginsenoside Re almost reversed the expression of ZO-1, E-cadherin, and occludin among the tight junction proteins. In contrast, tight junction disruption by HRV 16 was not effectively inhibited by treatment with ginsenoside Rb1and Rc (Fig. 2C–F).

3.3. Effects of ginsenoside Re on TEER in the HNE cells

To examine the potential effect of ginsenoside components in TEER reduction induced by HRV 16, we treated HNE cells with ginsenosides Re, Rb1, and Rc. As shown in Fig. 3, the treatment of ginsenoside Re prevented HRV 16-induced TEER reduction in HNE cells. In particular, at 100 μg/mL, ginsenoside Re almost reversed the decrease of TEER. In contrast, TEER reduction by HRV 16 was not effectively inhibited by treatment with ginsenoside Rb1and Rc.

3.4. Effects of ginsenoside Re on ROS production

Next, to investigate the effect of ginsenoside components in ROS generation induced by HRV 16, we treated HNE cells with ginsenosides Re, Rb1, and Rc. Treatment with 100 μg/mL ginsenoside Re prominently prevented HRV 16-induced ROS generation in HNE cells (Fig. 4A and B). We also investigated whether ginsenosides Rb1 and Rc inhibited HRV16-induced ROS production. As shown in Fig. 5A and B, ROS generation by HRV 16 was not efficiently inhibited by treatment with ginsenosides Rb1 and Rc in HNE cells. In addition, as shown in the fluorescence microscopy results in Fig. 5C, treatment with 100 μg/mL ginsenoside Re almost completely abolished the production of intracellular ROS in HNE cells. In contrast, HRV-induced ROS production was not inhibited by treatment with ginsenosides Rb1 and Rc.

3.5. ROS-dependent phosphatase inhibition and increase of phospho-tyrosine protein levels

Our previous study demonstrated that HRV 16-induced ROS is responsible for the increase in protein phosphorylation via the inhibition of phosphatases [21]. Therefore, we investigated the inhibitory effect of ginsenoside Re on the inhibition of ROS-dependent phosphatase and increase in phosphorylated tyrosine protein levels by HRV16. As shown in Fig. 6A, HNE cells incubated with HRV 16 showed significantly reduction in phosphatase activity as compared to the cells incubated with medium alone. Treatment with 100 μg/mL ginsenoside Re effectively recovered phosphatase inhibition in HNE cells infected with HRV 16, similar to DPI. Next, we investigated the inhibitory effect of ginsenoside Re in the increase in phospho-tyrosine protein levels induced by HRV 16. As shown in Fig. 6B, treatment with 100 μg/mL ginsenoside Re prominently prevented HRV 16-induced the increase in phospho-tyrosine band levels in HNE cells.

4. Discussion

Ginsenosides are considered key components of ginseng that functions against a variety of diseases. Among ginsenosides, Re is known to have anti-inflammatory, antioxidant, and antiviral effects [27–29]. In this study, we found that ginsenoside Re could restore disruption of tight junction in HNE cells by preventing ROS-mediated phosphatase inactivation by HRV 16.

Tight junctions form the intercellular barrier, which acts as the physical barrier against allergen and pathogen infection, and maintains homeostasis in epithelial cells [30]. Human nasal epithelial tight junctions maintain function by cytoplasmic tight junction proteins, including ZO-1, and junctional adhesion molecules (JAMs), occludin, and claudins [31]. A recent study reported that nasal epithelial barrier disruption was caused by the downregulation of ZO-1, occludin, E-cadherin, and claudin-1 expression by human

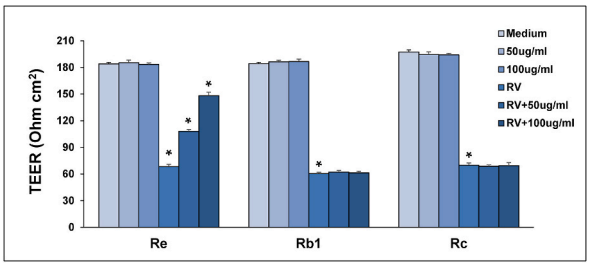

Fig. 3. Effect of ginsenoside components on TEER of HNE cells induced by human rhinovirus 16 (HRV 16). HNE cells were incubated for 72 h at 37 °C with or without HRV 16 in the absence or presence of ginsenoside Re, Rb1, and Rc (50 μg/mL and 100 μg/mL). TEER values for individual transwells derived from HNE cells infected with HRV 16 are shown. The background signal was subtracted (using TEER values from a blank Transwell), and data were normalized to controls.

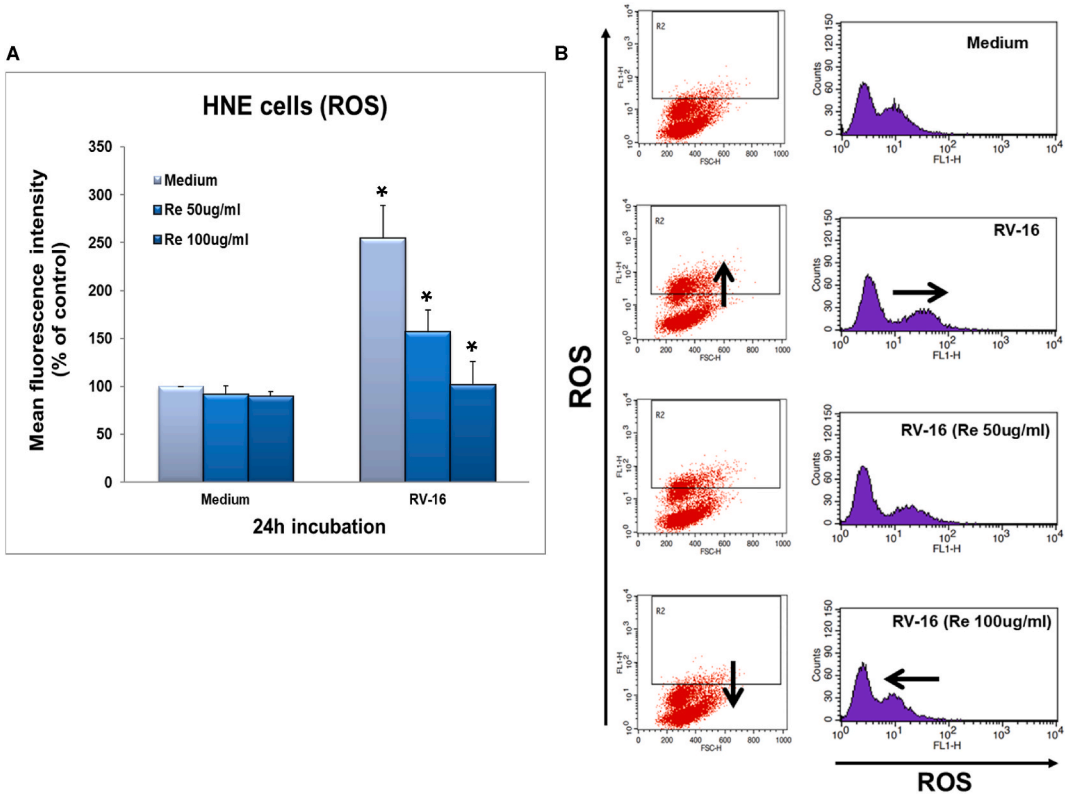

Fig. 4. A, B. The effect of ginsenoside Re on ROS generation induced by HRV 16. HNE cells were co-incubated for 24 h with HRV 16 in the absence or presence of ginsenoside Re (50 and 100 μg/mL) and the intracellular ROS production was analyzed by flow cytometry. Data are presented as the means ± SEM from three independent experiments. *, $P < 0.005$.

rhinovirus 16 [21]. Herein, we established tight junction disruption, characterized by a decrease in the expression of tight junction proteins claudin-1, ZO-1, E-cadherin, and occludin in HNE cells infected with HRV 16, and the results are also supported by our previous study demonstrating that HRV 16 infection led to tight junction disruption in human nasal epithelial cells [32].

P. ginseng has been a traditional medicine used in Korea and China for more than 5000 years [33]. Steamed and fermented ginseng produces red ginseng with a chemical composition that is different than the original ginseng. Many saponins, including ginsenoside, have been shown to have various useful effects, including antiviral activity. There are reports that ginsenosides cause adjuvant effects when used in combination with several vaccines, including influenza and parvovirus vaccines [7,34]. However, there are no previous reports on the antiviral activity of ginsenosides against human rhinovirus.

Ginsenoside Re has many protective effects on skin barrier function, such as ameliorating cornified cell envelope formation, filaggrin levels, caspase-14 activity [28], and downregulation of UVB-induced oxidative stress [35]. In the present study, we confirmed that ginsenoside Re restores tight junction protein expression by upregulating the tight junction-related proteins claudin-1, ZO-1, E-cadherin, and occludin in HRV16-induced nasal epithelial tight junction dysfunction.

After confirming the tight junction protection properties of ginsenoside Re, we focused on elucidating the underlying molecular mechanisms.

According to our previous study, HRV16 inhibits tight junction protein expression levels by producing ROS in human nasal epithelial cells [21]. We further investigated how ginsenoside Re inhibits tight junction disruption by focusing on ROS signaling. Our data exhibited that ginsenoside Re significantly reduced ROS production in HRV16-induced HNE cells, which may disrupt downstream signal transduction.

ROS are considered important components of many disorders and have been studied in various disease models. Ginsenoside Re demonstrates antioxidative properties through the upregulation of some antioxidant components, including total GSH and SOD, in HaCaT keratinocytes [36]. Re also prevents oxidative stress in cardiomyocytes [14]. Oxidative stress has been reported to regulate protein tyrosine phosphorylation by inhibiting phosphatase activity, resulting in Caco2 and T84 cells [37]. In particular, the tyrosine phosphorylation of proteins controlled by protein tyrosine phosphatases (PTPs) plays a crucial role in the pathogenesis of various disorders [38]. Therefore, regulation of PTPs is very important for cell signaling. To further analyze whether ginsenoside Re is involved in tyrosine phosphatase inactivation, we used ginsenoside Re and the ROS inhibitor DPI in HNE cells. Previous reports have shown that DPI inhibits ROS and is widely used in mechanistic studies. It can be inferred that the test compound plays a biological function by inhibiting ROS if the compound shows an inhibitory effect similar to that of DPI [21]. In this study, HRV16-induced inhibition of

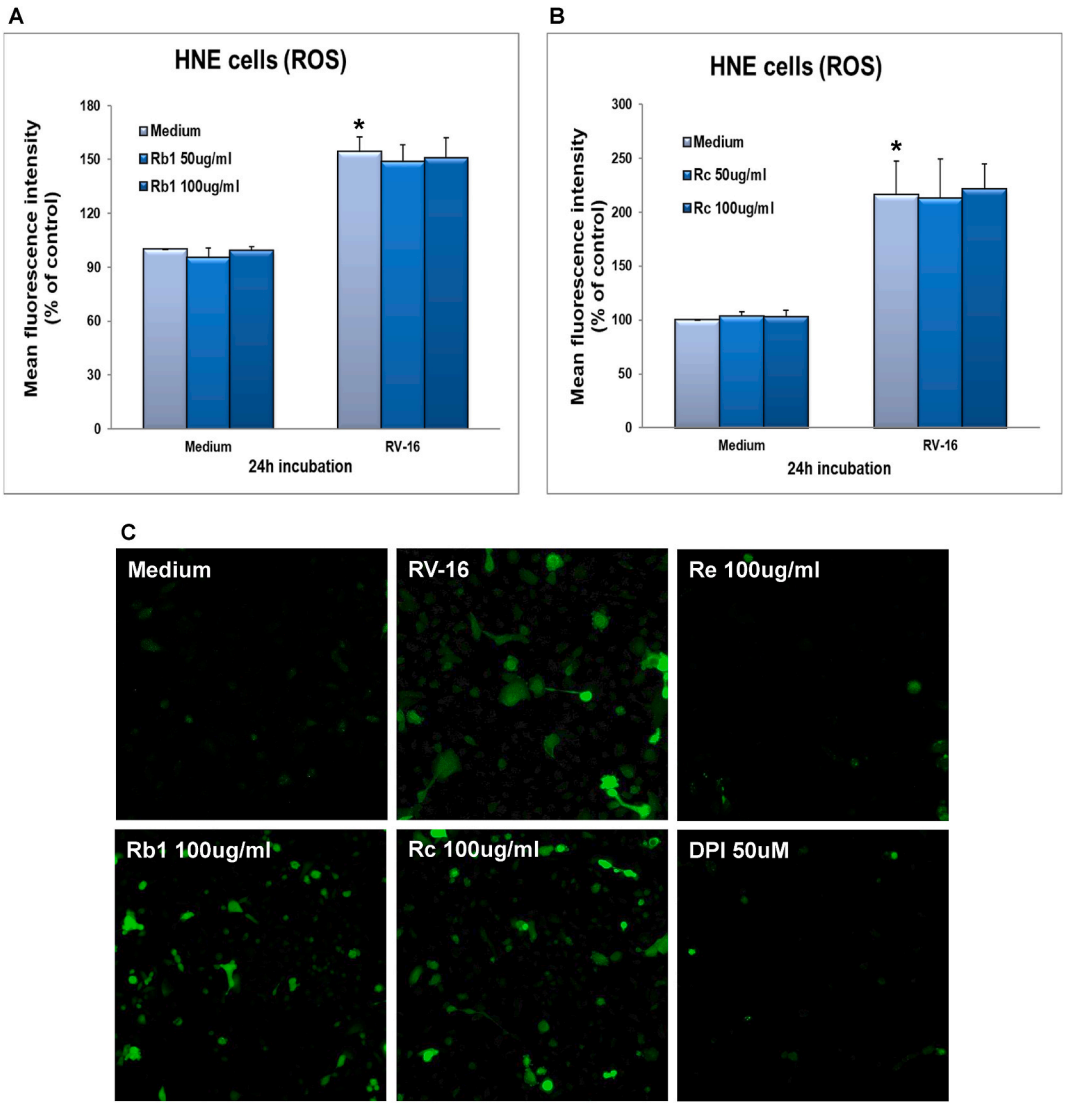

Fig. 5. A, B. The effect of ginsenoside Rb1 and Rc on ROS generation induced by HRV 16. HNE cells were co-incubated for 24 h with HRV 16 in the absence or presence of ginsenoside Rb1 and Rc (50 and 100 μg/mL) and the intracellular ROS production was analyzed by flow cytometry. Data are presented as the means ± SEM from three independent experiments. *, $P < 0.005$. C. Visualization of intracellular ROS accumulation in HNE cells incubated with HRV 16. HNE cells were co-incubated for 24 h with HRV 16 in the absence or presence of ginsenoside Re, Rb1, and Rc (50 and 100 μg/mL). The production of intracellular ROS in HNE cells was observed by inverted fluorescence microscopy (magnification × 200).

tyrosine phosphatases was reversed in the presence of the ginsenosides Re and DPI. The results showed that Re and DPI blocked the inhibitory effect of HRV16 on phosphatase activity, indicating that phosphatase activity was regulated by ROS.

We reported ROS is involved in tight junction disruption of human nasal epithelial cells induced by HRV16 in a previous paper [21]. In that report, the causal relationship between ROS, phosphatase activity, and p-tyr was revealed, and based on that study, the inhibitory effect of ginsenoside Re was confirmed. In the present study, we also found that ginsenoside Re blocked the increase in phospho-tyrosine protein levels and phosphatase inhibition, as well as ROS generation and tight junction disruption in HRV 16-infected human nasal epithelial cells. In particular, Re inhibited tight junction disruption and ROS generation, whereas Rc and Rb1 failed to suppress both.

In conclusion, we demonstrated for the first time that ginsenoside Re inhibits HRV16-induced tight junction disruption by inactivating ROS-mediated phosphatases in HNE cells (Fig. 7). Further studies on the detailed regulatory role of ginsenoside Re in this process will be helpful in obtaining a better understanding of HRV-induced inflammatory responses in HNE cells and facilitate the development of novel therapeutic strategies.

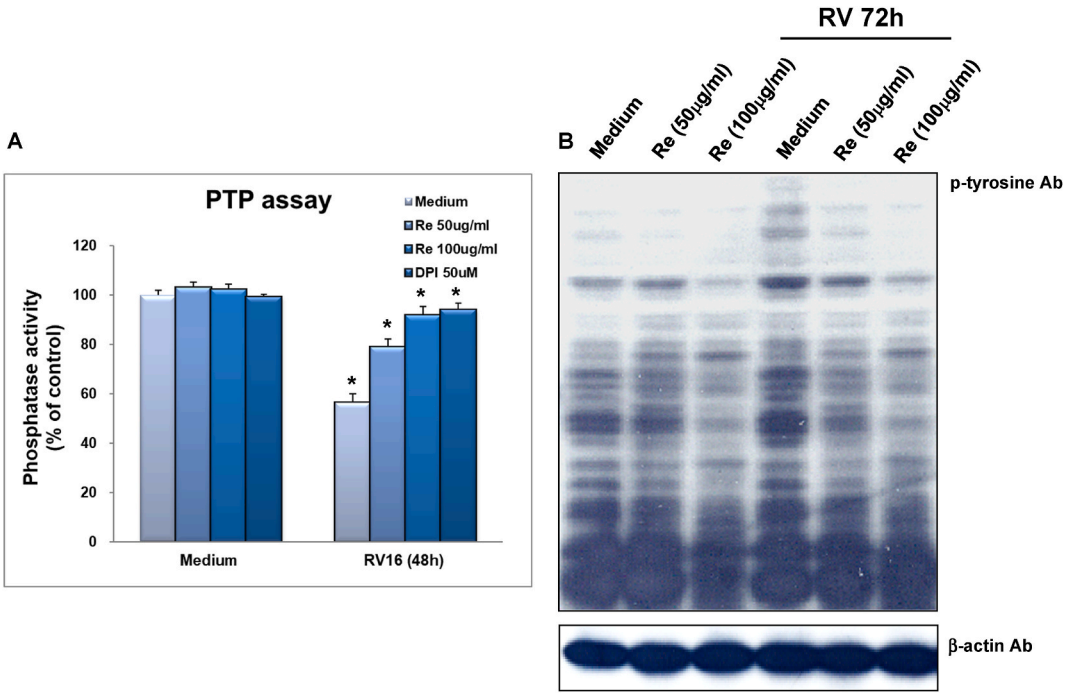

Fig. 6. Ginsenoside Re inhibits the HRV 16-induced inactivation of phosphatases and phosphorylation of protein tyrosine. HNE cells were incubated for 48 and 72 h at 37 °C with or without HRV 16 in the absence or presence of ginsenoside Re (50 μg/mL and 100 μg/mL). A. After incubation, PTPase activity was measured as described in Materials and Methods. Data are presented as the mean ± SEM from three independent experiments. *, $P < 0.005$. B. Whole-cell lysates were subjected to SDS-PAGE and probed with anti-phospho-tyrosine antibody. The figure is representative of three experiments showing similar results.

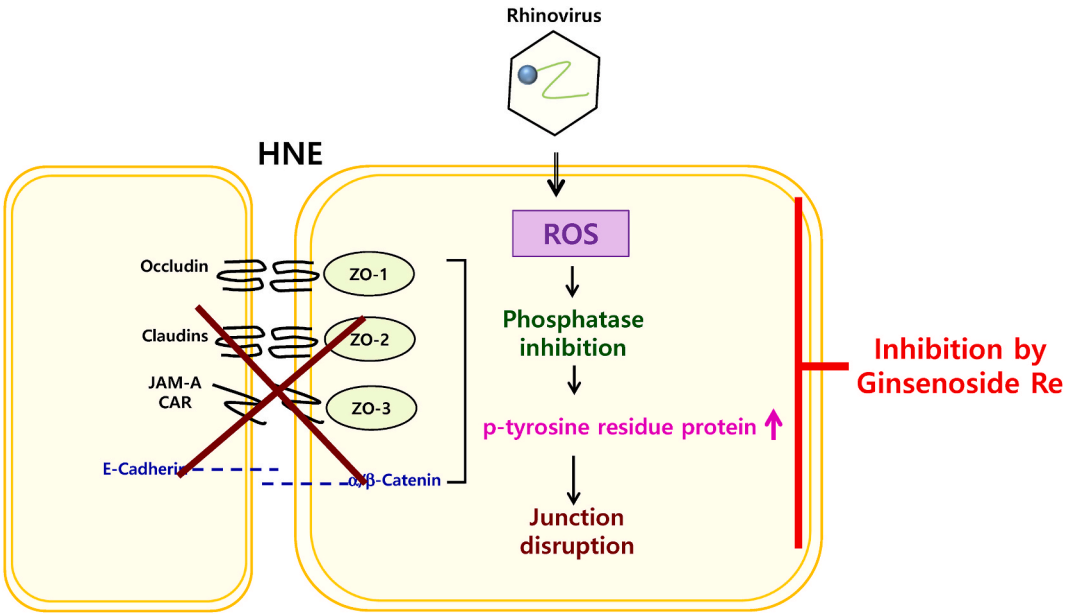

Fig. 7. Schematic diagram of effects of Ginsenoside Re on tight junction disruption in human nasal epithelial cells induced by HRV 16. After rhinovirus infection to nasal epithelial cells, there is a rapid increase in intracellular ROS. The generated ROS then extensively inactivates phosphatases. This inhibition of phosphatases results in marked increases in protein tyrosine phosphorylation, and the increase in phospho-tyrosine proteins contributes to the disruption of tight junction proteins. Ginsenoside Re can inhibit tight junction disruption through ROS suppression and phosphatases recovery.

Data availability statement

All authors had full access to all data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. These data are available to anyone upon request.

CRediT authorship contribution statement

Kyeong Ah Kim: Writing – original draft. **Joo Hyun Jung:** Data curation. **Yun Sook Choi:** Methodology. **Seon Tae Kim:** Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Seon Tae Kim reports financial support was provided by Korea Health Industry Development Institute. Seon Tae Kim reports a relationship with Korea Health Industry Development Institute that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by a grant from the Korea Health Technology R&D Project, which is funded by the Ministry of Health and Welfare, Republic of Korea (grant number: HI16C2319), and administered through the Korea Health Industry Development Institute (KHIDI).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27688.

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