Original Article
Korean red ginseng attenuates rhinovirus-stimulated IL-8, IL-6 responses in human nasal epithelial cells

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Abstract: Korean red ginseng (KRG) has multiple biological activities, including antiviral effects. Here, we investigated the protective effects of KRG on human rhinovirus (HRV) infection in primary human nasal epithelial cells (HNE). After HRV infection and subsequent treatment with KRG, the mRNA and protein levels of the inflammatory cytokines interleukin (IL)-8 and IL-6 were measured by real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. Viral titer and Western blot assays of rhinovirus 3C protease were used to evaluate viral replication in HRV-infected cells. Western blot analysis of the expression of proteins related to NF-κB and MAP kinase was used to assess the mechanisms underlying the effects of KRG on HRV-induced responses. KRG significantly reduced the HRV-induced upregulation of IL-8 and IL-6 mRNA and protein levels in primary HNE cells. Moreover, KRG treatment decreased viral replication in HRV-infected primary HNE cells. KRG treatment inhibited rhinovirus-induced NF-κB and MAP kinase activation. These results suggested that KRG treatment could attenuate the inflammatory responses to HRV infection, and thus may be used to prevent HRV-induced asthma exacerbations.

Keywords: Korean red ginseng, human rhinovirus, IL-8, IL-6, human nasal epithelial cells, NF-κB, MAP kinase

Introduction

Human rhinovirus (HRV), a member of the Picornaviridae family of viruses, is responsible for at least 50% of common colds; it also causes acute lower respiratory tract illnesses, including bronchiolitis and pneumonia [1-3]. In addition, HRVs exacerbate bronchial asthma and chronic obstructive pulmonary disease [4, 5]. HRV is a non-enveloped, positive, single-stranded RNA virus, of which more than 100 serotypes have been identified [6]. The major groups of HRV, constitute 90% of the HRV, bind to ICAM-1 receptor, whereas minor groups of HRV attach to proteins of LDL receptor family. Despite these differences, both the HRV group induces a similar chemokine release in human airway epithelial cells [7, 8].

Until now, there are reported several drugs having antiviral effect on human rhinovirus infection. Among several drugs, IFN-α was shown to be effective against Human rhinovirus infections. However, candidates treated with this drug experienced some side effects, and indi-cated resistance to the drug [9]. Also, there are potentially other substances such as Pleconaril and Iota-Carrageenan that may lead to the development of drugs to prevent human rhinovirus [10, 11]. But, its effects in treating rhinovirus infections are to be further studied.

Korean red ginseng is a traditional medicine used widely in Asia. KRG has various biological activities, including antitumor, anti-inflammatory, and anti-stress effects [12-14]. KRG exerts an antiviral effect against diverse RNA and DNA viruses, including human norovirus [15], respiratory syncytial virus [16], hepatitis A [17], influenza A [18], and herpes simplex virus [19], both *in vitro* and *in vivo*. The efficacy of KRG may be related to its modulation of the transcription factor NF-κB [20]. NF-κB activation mediates the production of cytokines and chemokines in response to Toll-like receptor recognition of intermediate dsRNA during viral infection [21, 22].

The human airway epithelium is the principle target of rhinovirus. HRV infection of the upper

respiratory tract is followed by infection of the lower respiratory tract [23]. Normal epithelial cells infected by HRV causes the innate immune and physiological response [24]. Furthermore, rhinovirus infection induces the production of inflammatory cytokines and chemokines (IL-6, IL-8, RANTES, and IL-10) in vivo and in vitro [25-27].

To date, no study has investigated the effect of KRG on inflammatory mediator production in HRV-infected airway epithelial cells. In this study, we examined the effect of KRG treatment on HRV-induced inflammatory responses in nasal epithelial cells, and evaluated its ability to suppress production of infectious virus.

Material and methods

Cell culture

Human nasal mucosa tissues were obtained from turbinoplasty patients who underwent inferior nasal turbinectomy. This study was approved by the Institutional Review Board of Gachon University Gil Medical Center (The approval number: GCIRB2014-362). Primary human nasal epithelial cells (HNE) were isolated by digesting the inferior turbinate with 1% pronase in phosphate-buffered saline (PBS) containing antibiotics for 1 h at 37°C. The cells were cultured in serum-free bronchial epithelial basal medium (BEBM; Lonza, Walkersville, MD, USA) supplemented with 0.5 µg/mL hydrocortisone, 5 µg/mL insulin, 10 µg/mL transferrin, 0.5 µg/mL epinephrine, 6.5 µg/mL triiodothyronine, 50 µg/mL gentamicin, 50 µg/mL amphotericin B, 0.1 ng/mL retinoic acid, and 0.5 ng/mL epidermal growth factor (Lonza Walkersville), bovine pituitary extract [1% (v/v)], and 1.5 µg/mL bovine serum albumin in a humidified, 5% CO_2 incubator at 37°C. Cells were grown to confluence (~2 weeks) and passaged a further two times before use in experiments.

HRV 16 infection and KRG treatment

HRV 16 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Viral stocks were prepared by infection of HeLa cells (ATCC) at 33°C for 7 d. Aliquots of virus prepared by polyethylene glycol (PEG) precipitation were frozen at -80°C after determination of viral titer. Passage 2 primary nasal epithelial cells were used in experiments. For HRV infection, cells were washed with PBS and infected with HRV 16 at 5×10^5 $TCID_{50}$/mL for 4 h in culture medium without fetal bovine serum (FBS) to allow for virus absorption. After incubation for 4 h at 33°C, the cells were washed with PBS and incubated in fresh medium. KRG or PBS was added at the required concentration for a further 24 h at 33°C. In some experiments, cells were simultaneously treated with HRV and KRG at 33°C for 1 h. The KRG (Korean red ginseng) used in this study was obtained from the Korea Ginseng Corporation (Daejeon, Korea).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from HNE cells using TRIzol® reagent (Life Technologies, Rockville, MD, USA). RNA (1 µg) was reverse transcribed into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Primers for β-actin, IL-8, and IL-6 were used as described previously [28]. Real-time PCR assayswere performed in 384-well plates using a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). All reactions were performed in a 10 µL volume comprising 5 µL of 2 × SYBR® Premix Ex Taq™II (Takara, Shiga, Japan), 0.84 µL of cDNA, and 400 nM of each primer. PCR conditions were as follows: 95°C for 30 s, 40 cycles of 5 s at 95°C, 10 s at 60°C, and 30 s at 72°C. Changes in gene expression were assessed using the comparative Ct method. Relative IL-8 and IL-6 mRNA levels were normalized by subtracting their Ct values from that of the internal control gene β-actin (ΔCt). Data are fold differences from the control using the $2^{-\Delta\Delta Ct}$ method.

Quantification of cytokines by enzyme-linked immunosorbent assay (ELISA)

CXCL8/IL-8 and IL-6 were quantified in cell-free supernatants using an ELISA MAX™ Deluxe kit (BioLegend, San Diego, CA, USA) according to the manufacturer's protocol. Data are expressed as picograms per milliliter and were derived by extrapolation from a standard curve that was generated in parallel with each experiment.

Virus titration assay

Experimental supernatants were serially diluted in Eagle's Minimal Essential Medium (MEM) containing 10% FBS (Invitrogen, Rockford, IL,
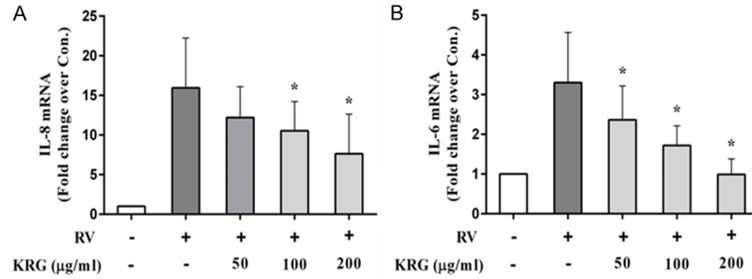
Figure 1. Effect of KRG on IL-8 and IL-6 mRNA levels in HRV 16-infected HNE cells. Addition of KRG at 24 h post-RV infection attenuated the HRV-stimulated IL-8 and IL-6 responses in primary HNE cells. IL-8 (A) and IL-6 (B) mRNA levels were determined by real-time RT-PCR. IL-8 and IL-6 expression was normalized to that of β-actin and expressed as fold change compared with control cells. Data are means and SD of 6-8 independent experiments (*$P < 0.05$, compared with rhinovirus-infected cells).
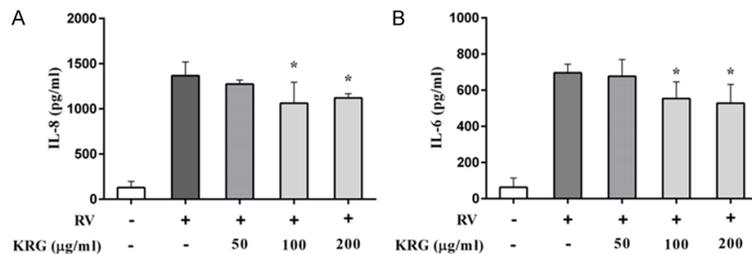
Figure 2. Effect of KRG on IL-8 and IL-6 secretion by HRV 16-infected HNE cells. KRG treatment attenuated HRV-stimulated IL-8 and IL-6 responses. Primary HNE cells were infected with rhinovirus, and then KRG was added. After 24 h, IL-8 (A) and IL-6 (B) levels in the medium were measured by ELISA. Data are means and SD of 6-8 independent experiments (*$P < 0.05$, compared with HRV-infected cells).

USA) and the supernatant was titrated against MRC-5 cells to determine the HRV $TCID_{50}$/mL. Following incubation at 33°C for 7 d, viral cytopathic effects were examined using an inverted microscope. Eight replicates of each dilution were assayed.

Western blotting

Total protein was prepared by dissolving cells in RIPA lysis buffer containing protease and phosphatase inhibitors. Nuclear proteins were extracted from cells using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were separated in 6-10% SDS-PAGE gels, and subsequently transferred onto PVDF membranes. Blots were blocked with 5% non-fat dried milk for 1 h, and incubated with the primary antibodies overnight at 4°C. Blots were developed using an ECL detection sys-

tem (Clarity™ Western ECL Substrate; Bio-Rad, Hercules, CA, USA).

Statistical analysis

The significance of differences among groups was assessed by a one-way analysis of variance (ANOVA), followed by Bonferroni's test or an unpaired Student's *t*-test. The results are presented as means ± standard deviation (SD) of independent experiments. A *P*-value < 0.05 was considered to indicate statistical significance.

Results

Effects of KRG on IL-8 and IL-6 mRNA levels

To evaluate the anti-inflammatory effect of KRG, cellular IL-8 and IL-6 mRNA levels at 24 h after KRG treatment post-RV infection were determined by real-time RTPCR. HRV infection resulted in significantly higher IL-8 and IL-6 levels compared with the control group (all *P* < 0.01). Treatment with KRG significantly reduced the IL-8 and IL-6 mRNA levels induced by HRV infection in a concentration-dependent manner (**Figure 1A** and **1B**).

Effects of KRG on IL-8 and IL-6 secretion

HRV infection resulted in a marked increase in IL-8 and IL-6 proteinlevels in the culture supernatant (all *P* < 0.01). KRG treatment (50 µg/mL) did not significantly affect the IL-8 and IL-6 levels (*P* > 0.05). However, KRG at 100 or 200 µg/mL significantly decreased the IL-8 and IL-6 protein levels in a concentration-dependent manner (**Figure 2**).

Effects of KRG on HRV replication

To determine whether the decreased IL-8 and IL-6 responses were due to a reduced viral load, viral titers were assayed. In cells infected
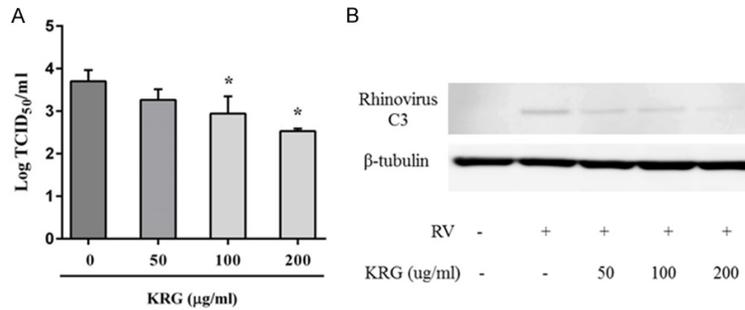
Figure 3. Effect of KRG on HRV 16 replication in HNE cells. Addition of KRG at 24 h after HRV infection resulted in decreased viral replication. After being subjected to the indicated treatments, supernatants of cell cultures were subjected to viral titer assays (A). Data aremeans and SD of three independent experiments (*P < 0.05, compared with HRV-infected cells). Total protein was isolated and subjected to Western blot analysis using an anti-rhinovirus C3 protease antibody (B). The result is a representative of three independent experiments.
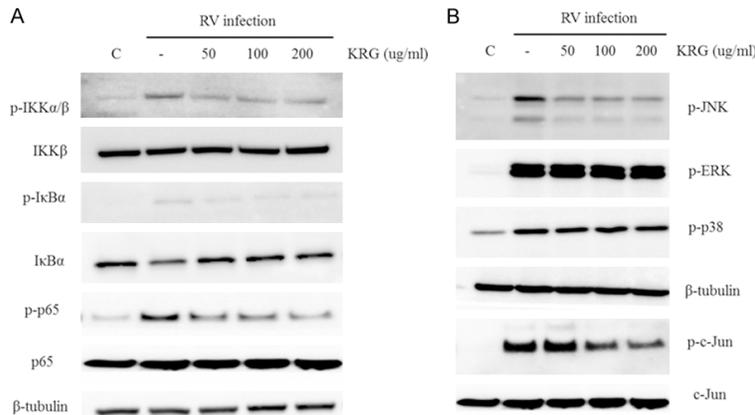
Figure 4. Effect of KRG on HRV 16-induced activation of NF-κB and MAPK in HNE cells. KRG treatment inhibited RV-induced NF-κB and MAPK activation. Total cell lysates or nuclear proteins were prepared at 1 h and levels of phosphorylated IKKα/β and IκBα, IKKβ, IκBα, p65, and β-tubulin (A) and of phosphorylated JNK, p38, ERK, c-Jun, and β-tubulin (B) were evaluated by Western blot assay using specific antibodies. Blots are representative of three independent experiments.

tease protein levels in a concentration-dependent manner (**Figure 3B**). Therefore, KRG may inhibit replication of HRV.

Effects of KRG on HRV-induced NF-κB activation

To determine the effect of KRG on RV-induced NF-κB activation, we assayed NF-κB protein levels by Western blotting. RV infection increased the phosphorylation of IKKα/β, phosphorylation and degradation of IκBα, and nuclear translocation of NF-κB p65. KRG treatment inhibited the HRV-induced NF-κB activation in a concentration-dependent manner (**Figure 4A**).

Effects of KRG on the HRV-induced MAPK signaling pathway

To determine the role played by MAPK signaling pathways in HRV-infected cells, MAP kinases (p38, ERK, and JNK) were examined. HRV infection significantly increased the levels of phosphorylated p38, ERK, and JNK. Interestingly, treatment of HRV-infected cells with KRG inhibited the activation of JNK, but not p38 or ERK, in a concentration-dependent manner. We next investigated whether HRV infection induced the phosphorylation of c-Jun/AP-1 and the effect of KRG thereon by Western blot analysis. HRV infection activated c-Jun/AP-1, and this effect was inhibited by KRG.

Discussion

Although the signaling pathways involved in HRV-induced inflammatory responses have been investigated in multiple cell lines, the effect of KRG in primary HRV infection of HNE cells has not yet been elucidated. This study shows for the first time that KRG inhibits HRV replication and inflammatory responses in primary HNE cells.

with HRV, the mean viral titer was $10^{3.7}$ TCID$_{50}$/mL. In HRV-infected cells treated with 50, 100, and 200 μg/mL of KRG, the viral titer was decreased to $10^{3.26}$ TCID$_{50}$/mL (P > 0.05), $10^{2.94}$ TCID$_{50}$/mL (P < 0.05), and $10^{2.53}$ TCID$_{50}$/mL (P < 0.05), respectively (**Figure 3A**). The protein level of rhinovirus C3 protease was assayed by Western blotting; rhinovirus C3 proteaseis encoded by HRV and plays an essential role in viral replication. As expected, rhinovirus C3 protease was not detected in the control group. KRG treatment markedly decreased the HRV-induced rhinovirus C3 pro-

In this study, HRV infection of HNE cells induced cytokine production, virus replication and activation of signaling molecules (NF-κB and MAP kinases). HRV-induced IL-8 and IL-6 production in HNE cells was significantly inhibited by pretreatment with KRG, suggesting an important role for KRG in the HRV-induced production of inflammatory mediators in nasal epithelial cells. Indeed, the amount of infective viral particles released from infected cells was significantly attenuated by a high concentration of KRG. Moreover, expression of rhinovirus 3C protease was also strongly inhibited by KRG. Our results show that KRG inhibits HRV-induced activation of NF-κB p65 and MAP kinase in nasal epithelial cells. Moreover, KRG at the highest concentration reduced NF-κB p65 and MAP kinase activation in HRV-infected HNE cells to levels similar to those in uninfected cells. Taken together, our results suggest that KRG plays a crucial role in the HRV-induced inflammatory responses of primary HNE cells during asthma or respiratory illness.

The effects of KRG treatment on the inflammatory responses of various cell types have been investigated [29-31]. However, most of these studies involved KRG components, such as saponin or KRG extracts. Ginseng has diverse biological activities, including anti-inflammatory and antitumor effects, and its major components are ginsenosides [32]. The antiallergy and anti-inflammatory effects of ginsenoside Rh1, the antiallergy effect of ginsenoside Rh2, and the antiallergy and anti-contact dermatitis activity of the saponin fraction of KRG and the ginsenosides Rg3, Rf, and Rh2 have also been reported. The anti-inflammatory effects of the saponin fraction of KRG and ginsenosides suggest their potential as anti-inflammatory agents [33, 34].

Epithelial cell lines including cultured primary epithelial cells produce various cytokines such as IL-8, IL-6, and GM-CSF following infection with HRV [27, 35-37]. Moreover, several of these cytokines was detected in nasal secretion during HPV infection in vivo [35, 36, 38, 39]. In this study, we demonstrated that KRG extracts significantly suppressed the HRV-induced elevation of IL-8 and IL-6 secretion by HNE cells.

IL-8 and IL-6 production of epithelial cells by rhinovirus infection could contribute to the inflammatory response. Expression in the epithelial cells of IL-6 and IL-8 by rhinovirus infection is mainly mediated by gene transcription, and induction of IL-6 and IL-8 is dependent upon activation of NF-κB [35, 40-43]. Activation of NF-κB plays a critical role in the expression of several cytokines including IL-8, IL-6, and GM-CSF according to the stimulation of agonists in a variety of cell types [44].

Recent studies have suggested that activation of NF-κB may also play a role in HRV-induced expression of IL-6 and IL-8 in epithelial cells [35, 40, 45]. These findings are consistent with the activation of NF-κB by other respiratory viruses [46]. A previous report indicatedthat NF-κB activation is involved in the regulation of the replication of viruses including papillomavirus 16 [47, 48] and influenza virus [49, 50]. Our findings suggest that infection with HRV increased the NF-κB p65 and p-IκBα protein levels in HNE cells (**Figure 4A**). Therefore, activation of NF-κB p65 in HRV-stimulated HNE cells influences IL-8 and IL-6 production.

It is not known the precise signal transduction pathways responsible for the production of cytokines by rhinovirus. A previous report demonstrated that p38 and c-Jun kinases regulate RANTES production in bronchial epithelial cells by influenza virus [51]. Several studies have demonstrated the role of JNK in the viral and host interactions. HIV-1 virus activates p38 and JNK MAP kinases through NF-κB and AP-1 signaling pathway [52]. Enterovirus 71 regulates IL-6 and IL-8 production by activating ERK1/2 and JNK1/2 [53]. We also investigated the possibility that other upstream signaling molecules activate c-Jun and thus induce IL-8 and IL-6 expression during HRV infection. Previous studies reported that the JNK plays an important role in the activation of c-Jun [54]. In addition, JNK is involved in activation of the transcription factors by a variety of stimuli. In this study, we demonstrated that KRG influences JNK and c-Jun expression during HRV infection. Pretreatment of HNE cells with KRG decreased the phosphorylation levels of JNK and c-Jun. These results demonstrate that KRG plays an important role in the HRV-induced expression of JNK and c-Jun, which might contribute to their anti-inflammatory effects.

Activation of MAP kinase and NF-κB p65 plays an important role in IL-8 and IL-6 production.

Therefore, we assumed that MAP kinase and NF-κB p65 activation in HRV-stimulated HNE cells influences IL-8 and IL-6 production. Our results are of particular importance since the induction of cytokines and activation of the MAP kinase/NF-κB p65 signaling pathway may be involved in the pathogenesis of HRV infection. However, the underlying molecular and cellular mechanisms of the antiviral effect of KRG on HRV remain to be elucidated.

In conclusion, we have demonstrated for the first time that KRG modulates HRV-induced inflammatory responses by activating NF-κB p65 and MAP kinase in HNE cells. Further studies on the regulatory role of KRG in this process will provide important information regarding the inflammatory responses to HRV infection, and facilitate development of novel therapeutic strategies.

Disclosure of conflict of interest

None.

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