Ginsenoside Rg3 attenuates skin disorders via down-regulation of MDM2/HIF1α signaling pathway

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ABSTRACT

Background: Thymic stromal lymphopoietin (TSLP) acts as a master switch for inflammatory responses. Ginsenoside Rg3 (Rg3) which is an active ingredient of Panax ginseng Meyer (Araliaceae) is known to possess various therapeutic effects. However, a modulatory effect of Rg3 on TSLP expression in the inflammatory responses remains poorly understood.

Methods: We investigated antiinflammatory effects of Rg3 on an in vitro model using HMC-1 cells stimulated by PMA plus calcium ionophore (PMACI), as well as an in vivo model using PMA-induced mouse ear edema. TSLP and vascular endothelial growth factor (VEGF) levels were detected using enzyme-linked immunosorbent assay or real-time PCR analysis. Murine double minute 2 (MDM2) and hypoxia-inducible factor 1α (HIF1α) expression levels were detected using Western blot analysis.

Results: Rg3 treatment restrained the production and mRNA expression levels of TSLP and VEGF in activated HMC-1 cells. Rg3 down-regulated the MDM2 expression level increased by PMACI stimulation. The HIF1α expression level was also reduced by Rg3 in activated HMC-1 cells. In addition, Rg3-administered mice showed the decreased redness and ear thickness in PMA-irradiated ear edema. Rg3 inhibited the TSLP and VEGF levels in the serum and ear tissue homogenate. Moreover, the MDM2 and HIF1α expression levels in the ear tissue homogenate were suppressed by Rg3.

Conclusion: Taken together, the current study identifies new mechanistic evidence about MDM2/HIF1α pathway in the antiinflammatory effect of Rg3, providing a new effective therapeutic strategy for the treatment of skin inflammatory diseases.

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1. Introduction

Inflammation is associated with numerous skin diseases [1–3]. Inflammatory reactions are characterized by swelling, heat, pain, and redness. Moreover, inflammation is regarded as an important factor of organ dysfunction which requires pharmacological intervention [4].

Various cells such as macrophages, keratinocytes, T cells, B cells, monocytes, eosinophils, neutrophils, and basophils are associated with the inflammatory reactions [5–7]. Mast cells also play roles in the inflammatory reactions [8]. Young et al. [9] reported that mast cells enhanced inflammation in chronic nonbacterial osteomyelitis and mast cell deficiency showed decreased inflammatory reactions in mice. Mast cell-depleted mice showed reduced joint inflammation in arthritis models [10]. Furthermore, mast cells ablation inhibited ear swelling response induced by 2,4-dinitrofluorobenzene (DNFB) in mice [11].

Thymic stromal lymphopoietin (TSLP) is crucial in the pathogenesis of skin inflammatory disorders such as contact dermatitis and atopic dermatitis. Recombinant TSLP treatment in the nape of the neck elevated scratching frequency in mice [12]. TSLP produced by mast cells plays a role in skin inflammatory responses [13].
Wong and colleagues [14] reported that TSLP acts as a master switch for inflammatory reactions by means of mast cell activation. TSLP was produced via murine double minute 2 (MDM2) signaling in macrophages [15]. Because MDM2 induces tissue inflammation, blockade of MDM2 would show potent antiinflammatory effects [16]. MDM2 induces activation of hypoxia-inducible factor 1α (HIF1α) [17]. Our previous study showed that treatment with MDM2 inhibitor (nutlin-3a) resulted in downregulation of HIF1α, indicating that HIF1α is a downstream factor of MDM2 [15]. Jang et al. [18] reported that TSLP expression was assumed that inhibitions of MDM2 and HIF1α effects such as anticancer, antidiabetes, antivirus, antiinflammatory, antiosteoporotic, antiatherosclerosis, antiarthritic, antiangiogenic, and antioxidant [19–26]. In addition, Rg3 promoted beta-amyloid peptide degradation, suggesting a preventive effect on Alzheimer’s disease [27]. However, the beneficial effect and precise mechanism of Rg3 on TSLP level in mast cells have not been clearly elucidated.

We hypothesized that Rg3 has a regulatory effect by inhibiting the TSLP production level via blockade of MDM2-HIF1α signaling pathway in mast cells during inflammatory responses. Thus, we assumed that inhibitions of MDM2 and HIF1α expression levels by Rg3 would reduce TSLP levels in serum and ear edema in mice.

2. Materials and methods

2.1. Enzyme-linked immunosorbent assay (ELISA)

The levels of TSLP and vascular endothelial growth factor (VEGF) in HMC-1 cell supernatants, sera and ear tissue homogenates were detected according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA), as previously described [28]. Each cytokine level was quantified by analyzing the absorbance with a microplate absorbance reader (Versa Max, Molecular Devices, Sunnyvale, CA, USA).

2.2. Quantitative RT-qPCR

Total RNA was extracted with an easy-BLUE™ RNA extraction kit (iNtRON Biotech Inc., Seongnam, Korea) and mixed with chloroform. After centrifugation, the collected upper aqueous phase was mixed with isopropanol. After centrifugation, RNA was treated with DNA-free DNA extraction kit (iNtRON Biotech Inc., Seongnam, Korea) and mixed with chloroform. The RNA was reverse transcribed with a cDNA synthesis kit (Bioneer Corporation, Daejeon, Korea). The cDNAs were used for quantification of gene expression by quantitative real-time PCR (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), as previously described [29]. The primer sequences were shown in Supplementary Table 1. The mRNA expression levels of TSLP and VEGF were normalized to GAPDH for each sample. Samples were run in duplicate.

2.3. Western blot analysis

The harvested HMC-1 cells were lysed in cell lysis buffer (Invitrogen™, Carlsbad, CA, USA) for analyses of MDM2 and GAPDH or lysed in nuclear extraction reagent (Thermo Fisher Scientific) for analyses of HIF1α and Poly (ADP-ribose) polymerase (PARP) with minor modifications [30,31]. Lysates were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After proteins were transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA), the membranes were blocked with 5% bovine serum albumin diluted in phosphate buffered saline (PBS) with Tween 20 (Sigma Chemical Co.). The membranes were incubated overnight with primary antibodies against MDM2, GAPDH, HIF1α or PARP. GAPDH and PARP were used as loading controls. The washed membranes were incubated with horseradish peroxidase-conjugated secondary anti-mouse antibodies (all primary and secondary antibodies from Santa Cruz Biotechnology). An enhanced chemiluminescence solution (DogenBio Co., Seoul, Korea) was used for development. Blots were quantified by Image J software (National Institute of Health, Bethesda, MD, USA). The density of each target band was normalized to GAPDH or PARP.

3. Results

3.1. Rg3 attenuates TSLP levels in HMC-1 cells stimulated with PMACI

To investigate whether Rg3 would regulate TSLP levels, we first examined the effect of Rg3 on cell viability via an MITT assay. HMC-1 cells were pre-incubated with Rg3 (0.1, 1 and 10 μg/ml) of in the presence of PMMA plus calcium ionophore (PMACI) by referring to the study of [19]. As shown in Fig. 1A, Rg3 treatment up to 10 μg/ml did not decrease the cell viability. The final concentration of a vehicle control (DMSO) was < 0.025% and did not affect the assay (Fig. 1A). DEX (100 nM) which is a critical antiinflammatory agent [32], did not affect this assay and was used as a positive control (Fig. 1A). In addition, the treatment with Rg3 reduced β-galactosidase activity in activated HMC-1 cells (p < 0.05; Fig. 1B). Thus, the concentrations of 0.1, 1 and 10 μg/ml of Rg3 were used to assess the regulatory effect of Rg3 in HMC-1 cells. In our previous study [33], TSLP mRNA expression was reached its peak 5 h after PMACI stimulation. Thus, we activated HMC-1 cells for 7 h to measure TSLP production level. Rg3 inhibited the increase in TSLP production level in a concentration-dependent manner in activated HMC-1 cells (p < 0.001; Fig. 1C). A vehicle control did not have an effect on the TSLP production level in HMC-1 cells stimulated with PMACI (Fig. 1C). Thus, the following experiments were performed excluding the vehicle control group. Rg3 suppressed the mRNA expression level of TSLP increased by PMACI in a concentration-dependent manner (p < 0.001; Fig. 1D). The inhibitory effects of Rg3 on the production and mRNA expression levels of TSLP were similar to those of DEX (p < 0.001; Fig. 1C and D).

3.2. Rg3 attenuates VEGF levels in HMC-1 cells stimulated with PMACI

VEGF is associated not only with pathological angiogenesis, but also with various inflammatory diseases [34]. Thus, we evaluated a regulatory effect of Rg3 on VEGF production level in activated HMC-1 cells. In response to PMACI, VEGF production level increased in HMC-1 cells. Rg3 suppressed the increase in the VEGF production level in a concentration-dependent manner in HMC-1 cells stimulated with PMACI (p < 0.001; Fig. 1E). A vehicle control did not have an effect on the VEGF production in activated HMC-1 cells (Fig. 1E). Rg3 significantly reduced the mRNA expression level of VEGF increased by PMACI (p < 0.001; Fig. 1F). These inhibitory effects of Rg3 on the VEGF levels were similar to those of DEX (p < 0.001; Fig. 1E and F).

3.3. Rg3 down-regulates MDM2 signaling pathways in HMC-1 cells stimulated with PMACI

A previous study reported that Rg3 down-regulates NF-kB signaling pathway in activated HMC-1 cells [20]. MDM2 activates the NF-kB signaling pathway and acts as a co-transcription factor...
for NF-κB target genes [35]. Thus, we attempted to further investigate whether Rg3 would regulate the TSLP and VEGF levels via MDM2 signaling pathways. First, we pre-treated an MDM2 inhibitor, nutlin-3a, and activated HMC-1 cells with PMACI. The TSLP (p < 0.001) and VEGF (p < 0.001) production levels were significantly reduced by nutlin-3a treatment (Fig. 2A and B). The inhibitory effects of nutlin-3a on the production levels were similar to those of Rg3 (Fig. 2A and B). Next, we investigated whether MDM2 expression level would up-regulated by PMACI stimulation. As shown in Fig. 2C and D, MDM2 expression began to be significantly induced 1 h after PMACI stimulation (p = 0.007). Elevation of MDM2 expression was the most at 3 h time point (p = 0.011) following PMACI stimulation (Fig. 2C and D). Thus, we selected 3 h time point to analyze the regulatory effect of Rg3 on the MDM2 expression. Expectedly, Rg3 treatment significantly reduced the MDM2 expression level increased by PMACI stimulation (p < 0.001, Fig. 2E and F). DEX also significantly decreased the MDM2 expression level (p < 0.001, Fig. 2E and F).

3.4. Rg3 down-regulates HIF1α signaling pathways in HMC-1 cells stimulated with PMACI

MDM2 is a positive activator of HIF1α and VEGF [17]. Both of MDM2 and HIF1α are key modulators of VEGF pathways [36]. Thus, we treated nutlin-3a in HMC-1 cells to study whether HIF1α expression would be regulated through MDM2 signaling pathways. To analyze HIF1α expression level, HMC-1 cells were activated with PMACI for 4 h, referring to the report of [37]. The PMACI stimulation significantly induced a marked increase in the HIF1α expression level (Fig. 3A and B, p < 0.001). The increased HIF1α expression level was significantly suppressed by nutlin-3a (p = 0.006, Fig. 3A and B). Next, we investigated whether Rg3 would down-regulate HIF1α expression in activated HMC-1 cells. Rg3 significantly restrained the HIF1α expression level in activated HMC-1 cells (p = 0.004, Fig. 3C and D). DEX also significantly restrained the HIF1α expression level (p = 0.003, Fig. 3). Moreover, an HIF1α inhibitor, YC-1 significantly diminished the production levels of TSLP (p < 0.001) and VEGF (p < 0.001) in activated HMC-1 cells (Fig. 2A and B). The inhibitory effects of YC-1 on the production levels were similar to those of Rg3 (Fig. 2A and B).

3.5. Rg3 attenuates PMA-irritated ear edema

Inflammatory reactions can occur when vascular tissues are subjected to harmful irritation [38]. Based on the above findings, we used an in vivo model of PMA-irritated ear edema and further investigated the regulatory effect of Rg3 on inflammatory responses. An increase in ear thickness indicates the degree of inflammation responses [39]. The PMA irritation induced significant redness and increases in ear thickness (p < 0.05, Fig. 4A and Table 1). However, Rg3-administered mice showed the decreased redness and ear thickness when compared to PMA control mice (p < 0.05, Fig. 4A and Table 1). In addition, Rg3 significantly decreased the serum TSLP (p = 0.003) and VEGF (p = 0.044) levels increased by PMA irritation (Fig. 4B and C). The TSLP (p < 0.001) and VEGF (p = 0.004) levels in the ear tissue homogenate were also markedly reduced by Rg3 administration (Fig. 4D and E). DEX also significantly inhibited these responses (Fig. 4 and Table 1). Finally, to study the regulatory effect of Rg3 on MDM2-HIF1α pathways in the in vivo model of PMA-irritated ear edema, we analyzed the MDM2 and HIF1α expression levels in the ear tissue homogenate. As shown in Fig. 5, Rg3 significantly suppressed the MDM2...
and HIF1α (p = 0.002) expression levels increased by PMA irritation in the ear tissue homogenate. DEX also significantly decreased the MDM2 (p < 0.001) and HIF1α (p = 0.011) expression levels (Fig. 5).

4. Discussion

Newly synthesized cytokines are secreted to the extracellular space for leading to inflammation after mast cell activation [40]. This mast cell activation can be reproduced by means of chemical stimuli. Stimulation with protein kinase C activator PMA plus calcium ionophore A23187 led to release of various inflammatory cytokines from HMC-1 cells [41]. Critical inflammatory cytokines, TSLP and VEGF were also produced by PMACI [33,37,41]. Serum and skin lesions of patients with atopic dermatitis contained high levels of TSLP [42]. TSLP receptor deficiency led to decreased skin inflammation in mice [43]. TSLP deficiency resulted in reduced skin inflammation in mice [13]. Transgenic overexpression of VEGF in the skin resulted in features of skin inflammation [44]. Scaldaferri et al. [45] suggested that agents that block VEGF signaling could suppress inflammatory responses in patients with inflammatory disease. Our findings presented that Rg3 reduced the production
Fig. 3. Rg3 down-regulates HIF1α signaling pathways in HMC-1 cells stimulated with PMACI. HMC-1 cells (5 × 10⁶) were pre-incubated with (A) nutlin-3a, (C) Rg3 or DEX (100 nM) for 1 h and then activated with PMACI for 4 h without media change. The expression levels of HIF1α and PARP in nuclear extraction were detected by immunoblot assay. (B,D) Quantification of relative levels was expressed as HIF1α/PARP. ###p < 0.001 considered significant compared to PMACI-inactivated group; *p < 0.05 and **p < 0.01 considered significant compared to PMACI-activated group.

Fig. 4. Rg3 attenuates PMA-irritated ear edema. (A) Ears were photographed after analyzing the ear thickness. (B) TSLP and (C) VEGF levels in serum were measured by ELISA. (D) TSLP and (E) VEGF levels in the ear tissue homogenate were assessed by ELISA. The total protein levels were assayed with a bicinchoninic acid protein assay kit. ###p < 0.001 and ##p < 0.01 considered significant compared to blank group; *p < 0.05, **p < 0.01, and ***p < 0.001 considered significant compared to PMA control group.
and mRNA expression of TSLP and VEGF in HMC-1 cells (Fig. 1). Thus, we presume that Rg3 may be useful for us to prevent and/or treat skin inflammatory disorders.

In general, it has been known that NF-κB is a regulator of inflammation-related TSLP expression at transcription level [33, 46]. MDM2 is an upstream factor of NF-κB. MDM2 transcriptionally up-regulates NF-κB expression and activates NF-κB-mediated gene expression [47]. Intradermal injection of MDM2 siRNA significantly suppressed TSLP mRNA and protein levels in the skin of DNF4-applied mice [48]. Jang and colleagues [18] suggested that HIF1α also is an important transcription factor of TSLP. MDM2 is an upstream factor of HIF1α [15]. In the present study, Rg3 inhibited activation of MDM2 and HIF1α in HMC-1 cells (Figs. 2 and 3). Thus, we postulate that Rg3 might regulate skin inflammatory reactions through MDM2/HIF1α signal pathway. Inflammatory stimuli (i.e., PMACI stimulation) increased the activation of MDM2 as well as HIF1α. Furthermore, an MDM2 inhibitor (nutlin-3a) dose-dependently inhibited HIF1α activation levels. To our knowledge, this is the first study showing new signal cascade (MDM2/HIF1α) for TSLP expression in mast cells.

PMA-induced ear edema model is a well-established in vivo model to confirm in vitro results in many studies [1, 49–51]. Our findings presented that treatment with Rg3 ameliorated the ear swelling responses resulted from PMA irritation (Table 1) and reduced the levels of TSLP and VEGF as well as the activation of MDM2 and HIF1α in mice (Figs. 4 and 5). Thus, we assume that the antiinflammatory effects of Rg3 would be easily translated to the human skin inflammatory disease.

A recent study suggested that the no-observed-adverse-effect level (NOAEL) for Rg3 in dogs is 20 mg/kg [52]. Additionally, Li and colleagues [53] reported that the NOAEL value for Rg3 in rats is 180 mg/kg. Highest concentration of Rg3 in the present study is 10 mg/kg. Hence, we presume that 10 mg/kg of Rg3 would not be toxic to humans.

Conclusionally, we elucidated the beneficial effect and precise mechanism of Rg3 on TSLP production in activated mast cells and found that Rg3 suppressed the TSLP production through down-regulation of MDM2/HIF1α signaling pathway in an in vitro model of PMACI-stimulated HMC-1 cells and a PMA-induced mouse ear edema model of inflammation. Our findings provided the new mechanistic evidence about MDM2/HIF1α pathway in the antiinflammatory effect of Rg3, suggesting a new effective therapeutic strategy for the treatment of skin inflammatory diseases.

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Appendix A. Supplementary data

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References


