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Ginsenoside Rg3 inhibits tumor necrosis factor-α-induced expression of cell adhesion molecules in human endothelial cells

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Ginsenoside Rg3 (Rg3), one of the most effective ginseng saponins, has anti-inflammatory and anti-cancer effects. This study examined the effects of Rg3 on cytokine-induced expression of adhesion molecules, which is a key early event in atherogenesis. Rg3 treatment inhibited tumor necrosis factor-α (TNF-α)-induced protein and mRNA expression of two cell adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) in ECV 304 human endothelial cells. In addition, expression of two pro-inflammatory cytokines, TNF-α and interleukin-1β (IL-1β), was suppressed by Rg3. Reporter gene analyses revealed that minimal reporter activities of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) were blocked by Rg3 in a concentration-dependent manner. Taken together, these results indicate that Rg3 may have anti-inflammatory and anti-atherosclerotic activities in the vasculature, which is mediated partly by down-regulation of the expression of cell adhesion molecules and proinflammatory cytokines in endothelial cells.

1. Introduction

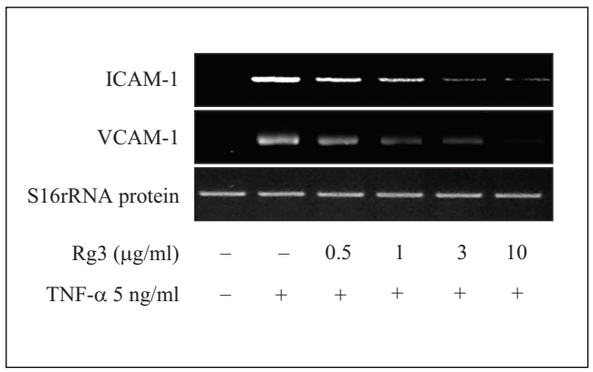
Atherosclerosis is a progressive vascular disease characterized by endothelial dysfunction, up-regulation of cell adhesion molecules, and accumulation of lipids, macrophages, smooth muscle cells (SMC) and fibrous tissue in the arterial intima. The process of atherogenesis is still incompletely understood, but it is generally believed that inflammation plays an important role in all stages of atherosclerosis (Ross 1999). Previous studies reported that activation of the vascular endothelium, adhesion of circulating monocytes to the injured endothelial layer, infiltration of monocytes into the vessel wall, and differentiation into macrophages are critical early events in the development of atherosclerosis (Price and Loscalzo 1999; Glass and Witztum 2001). Monocyte adhesion can be regulated by the expression of cell adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1). Moreover, expression of ICAM-1 and VCAM-1 is commonly up-regulated by proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (Pober et al. 1986; Marui et al. 1993).

Panax ginseng has been widely used as an herbal medicine in Asia for more than 2000 years and currently occupies an important place among the tonic remedies of oriental medicine. The diverse pharmacological effects of *P. ginseng* against cardiovascular disorders, immune responses and metabolic processes have been reported (Gillis 1997; Kim et al. 2005). Ginsenoside Rg3 (Rg3), a major biologically active component of *P. ginseng*, showed anti-hyperglycemic (Kim et al. 2009) anti-cancer (Keum et al. 2003) and anti-obesity activities (Hwang et al. 2009). We also found that Rg3 potently evoked endothelium-dependent relaxation via endothelial nitric oxide synthase (eNOS) activation in rat aorta (Kim et al. 1999).

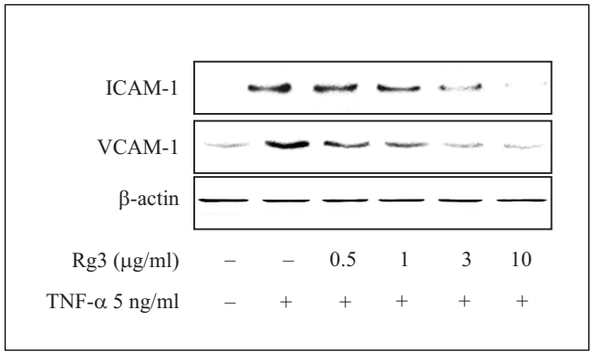
The effect of Rg3 on the expression of proinflammatory mediators has been estimated by several studies. Rg3 reduced expression levels of cyclooxygenase-2 (COX-2), IL-1β and interferon-γ induced by oxazolone applied to mouse ears (Bae et al. 2006a). In microglial cells, Rg3 and its metabolite Rh2 suppressed lipopolysaccharide/interferon-γ-induced COX-2 and TNF-α expression (Bae et al. 2006b). Although the aforementioned reports focused on anti-inflammatory effects of Rg3 in several cell types, the effects of Rg3 on the expression of adhesion molecules and proinflammatory cytokines in endothelial cells have not been clarified. Accordingly, in the present study, we determined whether Rg3 inhibits the expression of cellular adhesion molecules and proinflammatory cytokines in ECV 304 human endothelial cells and whether nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) are targets for the inhibitory actions of Rg3 against adhesion molecule expression.

2. Investigations, results and discussion

Initially, we determined the cytotoxicity of Rg3 to ECV 304 cells. Assays for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) revealed that Rg3 at concentrations up to 10 μg/ml did not significantly affect cell viability of ECV 304 cells (data not shown). Thus, we used Rg3 in the concentration range of 1-10 μg/ml for subsequent experiments. The increased expression of cell adhesion molecules such as VCAM-1, ICAM-1, and selectins on endothelial cells promotes the adhesion of monocytes, which is regarded as the molecular basis for the inflammatory response observed in various diseases (Ross 1999; Price and Loscalzo 1999). In particular, induction of VCAM-1 and ICAM-1 on the endothelial surface is considered an early event in atherogenesis (Ross 1999; Price and Loscalzo 1999). Hence, we first determined whether Rg3 inhibits the



(A)



(B)

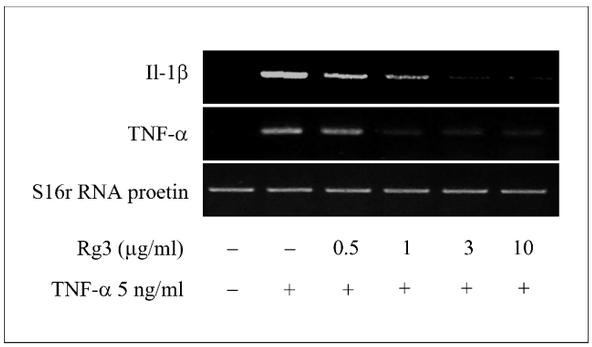
Fig. 1: Effects of Rg3 on TNF-α-induced expression of VCAM-1 and ICAM-1 protein and mRNA. The ECV 304 endothelial cells were pretreated with Rg3 (0.5–10 μg/ml) for 1 h and stimulated with 5 ng/ml TNF-α. (A) RT-PCR analysis. After 8 h incubation of cells with TNF-α, the total RNA was isolated and RT-PCR was performed. (B) Western blot analysis. After 6 h incubation, the cell lysates were blotted with the anti-ICAM-1, VCAM-1 and β-actin antibodies

expression of VCAM-1 and ICAM-1 in ECV 304 cells. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot analyses showed that Rg3 markedly inhibited TNF-α–induced mRNA and protein expression of ICAM-1 and VCAM-1 in a concentration-dependent manner (Fig. 1).

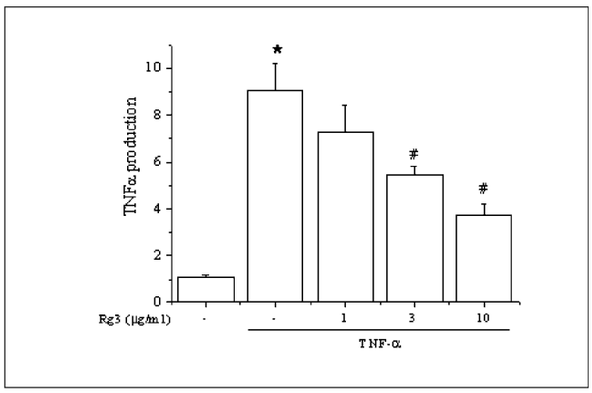
ICAM-1 and VCAM-1 are upregulated by proinflammatory cytokines, IL-1 and TNF-α (Pober et al. 1986; Marui et al. 1993). Clinical and basic studies have elucidated a potential link between proinflammatory cytokines and vascular occlusive diseases. Higher TNF-α levels correlate significantly with higher concentrations of atherogenic lipopoteins. Stimulation by this cytokine also causes higher production of reactive oxygen species, an important mediator of atherogenesis, via NADPH oxidase or ceramide-activated protein kinase in endothelial cells (Zhang et al. 2009). It has been also reported that IL-1β expression is enhanced in endothelial cells and macrophages of coronary arteries from ischemic hearts, compared to non-ischemic cardiomyopathic hearts (Glaea et al. 1996). The increased IL-1β is believed to be involved in the sustained inflammation that occurs during the development of atherosclerosis (Apostolakis et al. 2008). To examine the effect of Rg3 on the production of IL-1β and TNF-α in response to an inflammatory signal, ECV 304 cells were pretreated with Rg3 for 1 h and then stimulated with TNF-α. Fig. 2A shows that mRNA expression of both IL-1β and TNF-α are suppressed by Rg3. In addition, an enzyme-linked immunosorbent assay (ELISA) using a TNF-α specific antibody confirmed that protein production of TNF-α was also decreased by Rg3 (Fig. 2B). The data indicate that Rg3 inhibits the expression of proinflammatory cytokines and cell adhesion molecules.

NF-κB and AP-1 are key transcription factors implicated in the regulation of proinflammatory genes including VCAM-1, ICAM-1 and TNF-α (Iademarco et al. 1992; Collins et al. 1995; Iiyama et al. 1999). Functional analysis of the human VCAM-1 promoter showed that VCAM-1 gene transcription is regulated by various transcription factors, including NF-κB, GATA, AP-1 and interferon regulatory factor-1, in response to pro-inflammatory cytokines (Iademarco et al. 1992; Collins et al. 1995). In particular, the transcriptional activation of NF-κB is the most important process for the expression of pro-inflammatory cell adhesion molecules (Ledebur et al. 1995; Ross 1999). Moreover, Rg3 was reported to suppress the activities of NF-κB and AP-1 in glial cells and phorbol ester-applied skin tissues (Keum et al. 2003; Bae et al. 2006b). Hence, we determined whether NF-κB and AP-1 activities are inhibited by Rg3 in TNF-α exposed ECV 304 cells. Rg3 concentration-dependently blocked reporter activities of both NF-κB and AP-1 (Fig. 3). From these results, we conclude that Rg3's inhibition of the expression of adhesion molecules and proinflammatory cytokines is linked to its NF-κB and AP-1 blocking activities.

In our previous study (Kim et al. 2003), we found that long-term incubation of Rg3 decreases vasoconstrictor responsiveness in endothelium-denuded rat aorta, and further demonstrated that NF-κB activation and subsequent iNOS induction by Rg3 was involved in the decreased smooth muscle response. However, we found here that TNF-α-stimulated NF-κB activation and adhesion molecule expression were potently suppressed by Rg3. Rg3



(A)



(B)

Fig. 2: Inhibition of IL-1β and TNFα mRNA expression by Rg3. (A) RT-PCR analysis. The ECV 304 endothelial cells were pretreated with Rg3 (0.5–10 μg/ml) for 1 h and stimulated with 5 ng/ml TNF-α. After 8 h incubation, the total RNA was isolated and RT-PCR was performed. (B) TNF-α ELISA. ECV 304 cells were incubated in serum-free medium for 24 h. The endothelial cells were pretreated with Rg3 for 1 h and stimulated with TNF-α for 6 h. production of TNF-α was analyzed using TNF-α specific ELISA. The values are expressed as a relative ratio to untreated control. Data represents the mean ± SD (n = 9)(significant as compared to control, $^{*}p < 0.05$; significant as compared to TNF-α treated group, $^{\#}p < 0.05$)

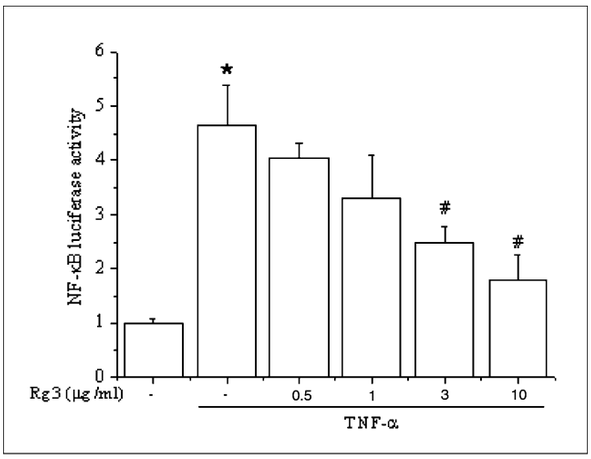


Fig. 3: Suppression of (A) NF-κB and (B) AP-1 activation by Rg3. ECV 304 cells were transiently transfected with NF-κB (pNF-κB-Luc) or AP-1 (pAP-1-Luc) minimal reporter gene. Following transfection, cells were treated with various concentrations of Rg3 (0.5–10 μg/ml) and 10 ng/ml TNFα for 24 h prior to lysis and measurement of NF-κB and AP-1 reporter activity by luciferase assays. Data represents the mean ± SD (n = 5)(significant as compared to control, $^{*}p < 0.05$; significant as compared to TNF-α treated group, $^{\#}p < 0.05$)

may have bi-directional activities on NF-κB activation. Stimulation of immune-responsive cells with Rg3 alone can mimic cytokine signaling. Rg3 co-exposure of cells with potent proinflammatory cytokines can also reduce inflammatory responses in the vasculature. Our finding has implications for the elucidation of the pharmacological mechanism of Rg3's beneficial effects on the cardiovascular system.

3. Experimental

Most reagents were purchased from Sigma (St. Louis, MO). ECV 304 cells were obtained from the American type culture collection (Bethesda, MD) and incubated at 37 °C in a 5% CO_2/95% air atmosphere in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Western blot analysis, ELISA, RT-PCR and reporter gene assays were done as previously described (Kim et al. 2009). PCR was done using selective primers for VCAM-1 (sense primer, 5′- ATGGTTGC TATGGCCG-GAAG -3′; antisense primer, 5′- GAACAGGTCATGGTCACAG-3′), ICAM-1 (sense primer, 5′-CCGGAAGGTGTATGAACTG-3′; antisense primer, 5′- CCGTGGCAATGAGACTCTGC-3′), TNF-α (sense primer, 5′- CCCAGGCAGTCAGATCATCTTC-3′ and antisense primer 5′-AGCTGCCCCTCAGCTTGA-3′), IL-1β (sense primer, 5′- AAGTACAAAAAGACTATGGTGCA-3′; antisense primer 5′-GATACGTTTTTGATCCTCAAGTGTGAAG-3′), and S16 ribosomal protein (S16r) genes (sense, 5′-TCCAAGGGTCCGCTGCAGTC-3′; antisense, 5′-CGTTCACCTTGATGAGCCCATT-3′). Student's *t*-test was used to examine inter-group differences. Statistical significance was set at either the $p < 0.05$ or < 0.01 level as indicated.

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