

Original Research Article

Ginsenoside Rb1 Reduces Nitric Oxide Production via Inhibition of Nuclear Factor- κ B Activation in Interleukin-1 β -Stimulated SW1353 Chondrosarcoma Cells

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Abstract

Purpose: To investigate the effect and the potential mechanisms of ginsenoside Rb1 on nitric oxide (NO) production in chondrocytes.

Methods: SW1353 chondrosarcoma cells were stimulated with interleukin-1 β (IL-1 β) in the presence of 20, 40, 80 μ M ginsenoside Rb1. NO concentration was assessed by the Griess reaction. Expression of inducible nitric oxide synthase (iNOS), content of inhibitor of NF- κ B (I κ B) α and nuclear level of nuclear factor (NF)- κ B p65 were determined by Western blot. DNA binding activity of NF- κ B was evaluated with Trans AM™ kit for NF- κ B p65.

Results: Ginsenoside Rb1 (40 and 80 μ M) significantly decreased NO level by 24 ($p < 0.05$) and 46 % ($p < 0.01$), as well as iNOS protein expression by 40 and 55 % ($p < 0.01$), respectively, in IL-1 β -stimulated SW1353 cells. Ginsenoside Rb1 (40 and 80 μ M) also markedly elevated I κ B α protein content by 200 and 260 % ($p < 0.01$), reduced the nuclear level of p65 protein by 30 and 40 % ($p < 0.01$), as well as decreased the DNA binding activity of NF- κ B by 40 and 50 % (both $p < 0.01$), respectively, in IL-1 β -stimulated SW1353 cells.

Conclusion: These results suggest that ginsenoside Rb1 inhibits IL-1 β -induced NO production through downregulation of NF- κ B-dependent iNOS expression in chondrocytes, and also underlines the potential mechanisms of ginseng activity in OA treatment of TCM.

Keywords: Ginsenoside Rb1, Nitric oxide, Nuclear factor- κ B, Chondrocytes, Osteoarthritis

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INTRODUCTION

Osteoarthritis (OA) is one of the most common chronic diseases affecting the elderly and is characterized by the abnormal degradation of the cartilage matrix and immoderate deposition of subchondral bone matrix. A growing body of evidence supports the fact that nitric oxide (NO) plays a role in the pathological development of OA [1]. Osteoarthritic joints exhibited elevated

NO production as well as increased amounts of other inflammatory mediators [2]. OA patients exhibited markers of enhanced NO production in their urine, serum, and synovial fluid [3]. In inflammatory reactions, NO is generated principally by inducible NO synthase (iNOS). Chondrocytes, the cellular occupants of cartilage and thus central to maintaining the integrity of the matrix, are the main cellular source of NO and iNOS generation in OA [4]. NO is considered as

a pro-inflammatory agent as well as a potent catabolic mediator in OA since it promotes the production of inflammatory cytokines [5], activates matrix metalloproteinases [6] and inhibits the synthesis of collagen and proteoglycan [7].

Nuclear factor- κ B (NF- κ B) is the key transcription factor regulating iNOS gene expression in inflammatory conditions [8]. NF- κ B exists as a homo- or hetero-dimeric form of Rel family proteins which include p65, p50, p52, RelB and cRel. The complex between p65/p50 is the predominant heterodimer. NF- κ B is sequestered in the cytoplasm where it is bound to inhibitors of NF- κ B (I κ B) such as I κ B α , I κ B β , I κ B γ and I κ B ϵ . Activation of NF- κ B is dependent on the phosphorylation and degradation of I κ B. Free NF- κ B can then translocate to the nucleus, bind to the specific DNA binding sites, and initiate expression of target genes including iNOS, interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) [9]. Concurrently, IL-1 β and TNF- α are also the potent inducers for NF- κ B activation [10].

Principal treatments for OA are mainly palliative for symptoms of dyskinesia and joint pain [11]. Use of ginseng dates back more than 2000 years in China, and is one of the most popular Chinese materia medica in traditional Chinese medicine (TCM) formulas for OA treatment [12]. Ginsenoside Rb1, a principle active constituent of ginseng, has been reported to have anti-inflammatory action. Ginsenoside Rb1 inhibited the expression of TNF- α , IL-1 β and IL-6 in lipopolysaccharide (LPS) -stimulated murine peritoneal macrophages [13]. Although release of NO by ginsenoside Rb1 may underlie the cardiovascular protection [14], the effects and mechanisms of ginsenoside Rb1 on NO production by chondrocytes during inflammatory conditions are not clear. Thus, the purpose of this study was to investigate the effect of ginsenoside Rb1 on NO production, iNOS expression and NF- κ B activation in IL-1 β -stimulated SW1353 chondrosarcoma cells.

EXPERIMENTAL

Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified. Ginsenoside Rb1 was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Recombinant human IL-1 β was purchased from R&D System (Minneapolis, Minnesota, USA). Dulbecco's

modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Antibodies against iNOS, I κ B α , NF- κ B p65, β -actin, and lamin B1 were purchased from Santa Cruz (Santa Cruz, CA, USA). ECL Western Blot detection system and polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). Trans AM™ kit for p65 was purchased from Active Motif (Carlsbad, CA, USA). NO detection kit based on the Griess reaction was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Cell culture

SW1353 human chondrosarcoma cell line purchased from the American Type Culture Collection were cultured in DMEM with 10 % FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37 °C in humidified air with 5 % CO₂. Cells were cultured in DMEM with 1 % FBS for 24 h, and then stimulated with 10 ng/mL IL-1 β for indicated time periods. The supernatant or the cell layer was then collected for further analyses.

MTT assay

MTT assay was used to evaluate the cytotoxic effect. Approximately 1×10^4 SW1353 cells/well were incubated in a total volume of 200 μ l in 96-well plates with or without ginsenoside Rb1. After an incubation period of 24 h, MTT was added for 4 h at the final concentration of 0.5 mg/ml. Subsequently, the culture medium was removed and after dissolving the formazan crystals in dimethyl sulfoxide (DMSO), plates were read immediately at 570 nm using an absorbance plate reader (Bio-Rad, USA). Wells containing incubation media without cells were used as control. SW1353 cells treated with vehicle only were defined as 100 % viable. Cell survival was defined as the growth of treated cells compared with untreated cells.

NO measurement

NO production in the supernatants was analyzed using a commercial NO detection kit based on the Griess reaction according to the manufacturer's instructions.

Preparation of protein extracts (total and nuclear)

The total cellular proteins were extracted by radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % NP-40, 0.1 % Sodium dodecyl sulfate (SDS),

0.5 % sodium deoxycholate) and ethylene diamine tetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche). To separate the nuclear proteins, cells were suspended in 250 μ l of buffer A (10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 % NP-40 and EDTA-free protease inhibitor cocktail), followed by centrifugation at 10000 g for 10 min at 4 °C. The pelleted nucleic protein extracts were resuspended in 50 μ l of buffer B (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate, and EDTA-free protease inhibitor cocktail) and centrifuged at 10000 g for 10 min at 4 °C. The quantity of proteins was measured using the Lowry method and all the protein samples were stored at -70 °C.

Western blot analysis

Equal amounts of total (for iNOS, I κ B α and β -actin) or nuclear (for P65 and lamin B1) protein samples were separated by 10 % SDS-polyacrylamide gelelectrophoresis (PAGE), and then transferred onto PVDF membranes. After blocking with 5 % nonfat milk in Tris-buffered saline with Tween (TBST) buffer (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1 % Tween 20) for 1 h at room temperature, PVDF membranes were incubated with the primary antibody at 4 °C overnight and subsequently with peroxidase-conjugated second antibody at room temperature for 1 h. The protein bands were detected with ECL. Signals were detected and analyzed using the ChemiDoc XRS imaging system (Bio-Rad, USA).

NF- κ B DNA-binding activity

The binding ability of NF- κ B to DNA consensus sequences was measured by ELISA in nuclear protein extracts using the Trans AM™ kit for p65 following the manufacturer's recommendations. Briefly, nuclear extracts (5 μ g) were added to the wells followed by the primary antibody against p65 and the horseradish peroxidase-conjugated secondary antibody. The optical density was measured at 450 nm with an absorbance plate reader (Bio-Rad, USA).

Statistical analysis

Results are reported as mean \pm SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Effect of ginsenoside Rb1 on NO production

We first tested whether ginsenoside Rb1 had any effect on IL-1 β -induced NO production. Basal levels of NO in SW1353 cells were low without IL-1 β stimulation. The concentration of NO increased more than 15-fold ($p < 0.01$) in presence of IL-1 β after 24 h compared to baseline levels (Fig 1A, B). This effect was abrogated in a concentration-dependent manner when SW1353 cells were coincubated with various concentrations of ginsenoside Rb1. Ginsenoside Rb1 at 40 μ M and 80 μ M decreased IL-1 β -induced NO production by approximately 24 % ($p < 0.05$) and 46 % ($p < 0.01$), respectively (Fig 1A). Ginsenoside Rb1 at 20 μ M did not show any effect on IL-1 β -induced NO production in SW1353 cells (Fig 1A). Ginsenoside Rb1 at the concentrations used in this study did not appear to be cytotoxic to the SW1353 cells (Fig 1B).

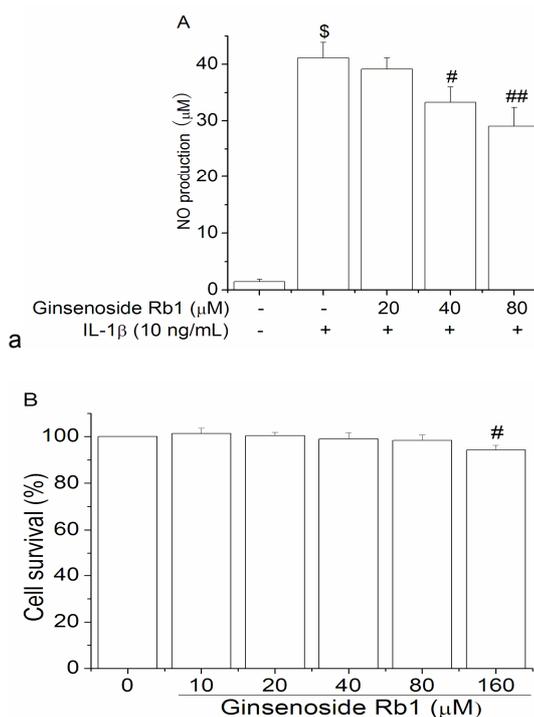


Figure 1: Effect of ginsenoside Rb1 on NO production in IL-1 β -induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μ M) for 2 h and stimulated with 10 ng/mL IL-1 β for 24 h. (A) Secreted NO in the cell-free culture media was measured by Greiss reaction. (B) MTT assay was performed to evaluate cytotoxicity. Three independent experiments performed in duplicate; values are mean \pm SD. In Figure 1A, $^{\$}p < 0.01$ vs. media alone-treated group, and $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ vs. IL-1 β alone-treated group. In Figure 1B, $^{\#}p < 0.05$ vs. media alone-treated group.

Effect of ginsenoside Rb1 on iNOS expression

Western blot analysis was carried out to investigate whether the inhibitory effect of ginsenoside Rb1 on NO production was due to its influence on iNOS synthesis, a dominant source of NO in inflammatory conditions. Expression of iNOS protein was not detectable in unstimulated SW1353 cells, but was considerably induced upon exposure to IL-1 β alone (Fig 2A, B). Ginsenoside Rb1 decreased IL-1 β -induced expression of iNOS protein in a concentration-dependent manner, corresponding to about 40 % inhibition at 40 μ M, 55 % at 80 μ M (both $p < 0.01$, Fig 2B). Levels of iNOS protein were not significantly affected at 20 μ M ginsenoside Rb1 (Fig 2A, B).

Effect of ginsenoside Rb1 on I κ B α degradation

Because the translocation of NF- κ B to the nucleus depends on degradation of I κ B α , we investigated whether ginsenoside Rb1 could affect this process. Reduction of I κ B α protein dramatically occurred within 30 min upon exposure to IL-1 β alone ($p < 0.01$, Fig 3A, B). Ginsenoside Rb1 at 40 μ M and 80 μ M markedly promoted the content of I κ B α protein in IL-1 β -induced SW1353 cells by approximately 200 % and 260 %, respectively (both $p < 0.01$, Fig 3B). However, ginsenoside Rb1 at 20 μ M did not have a significant effect on IL-1 β -induced I κ B α protein content (Fig 3A, B).

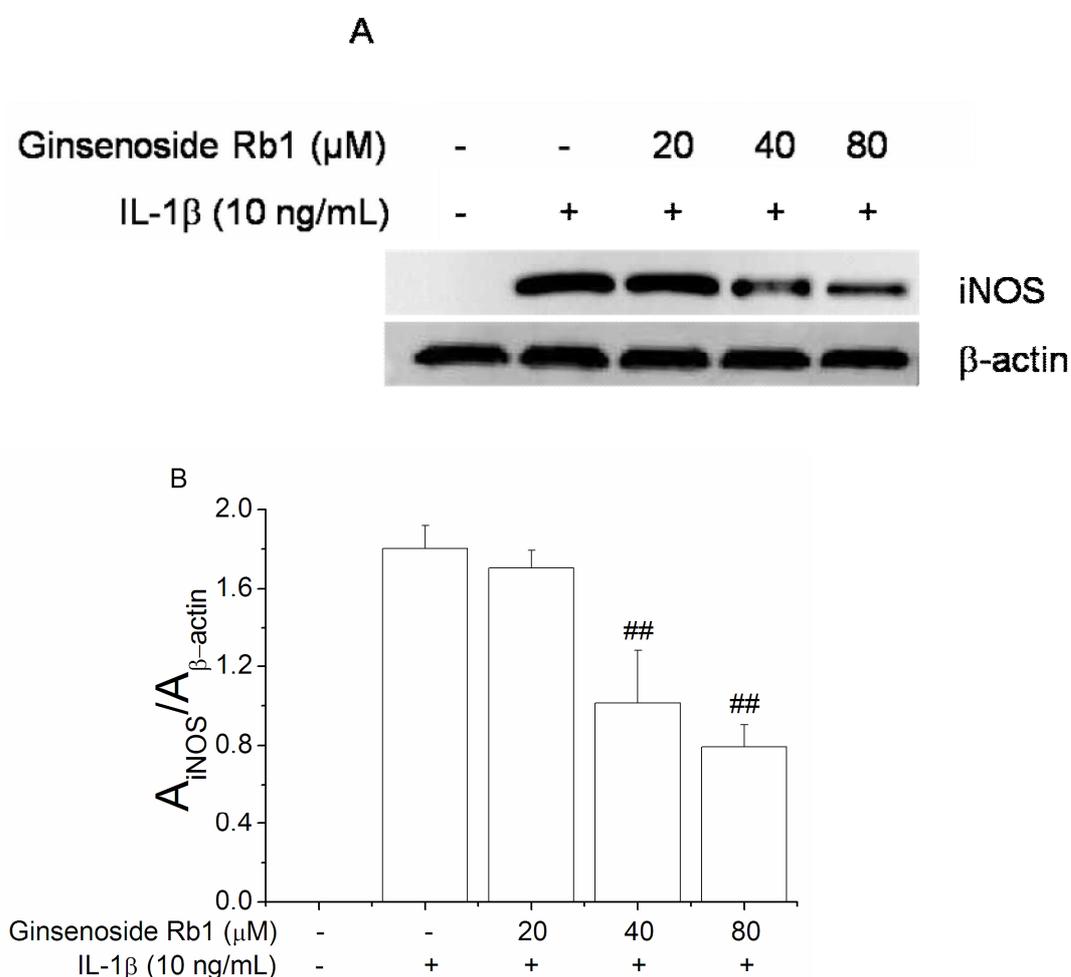


Figure 2: Effect of ginsenoside Rb1 on iNOS expression in IL-1 β -induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μ M) for 2 h and stimulated with 10 ng/mL IL-1 β for 24 h. (A) iNOS protein in the cell lysates was detected by Western blot; (B) Bar graphs showed quantitative evaluation of iNOS bands by densitometry. Three independent experiments performed in duplicate; values are mean \pm SD; ## $p < 0.01$ vs. IL-1 β alone-treated group

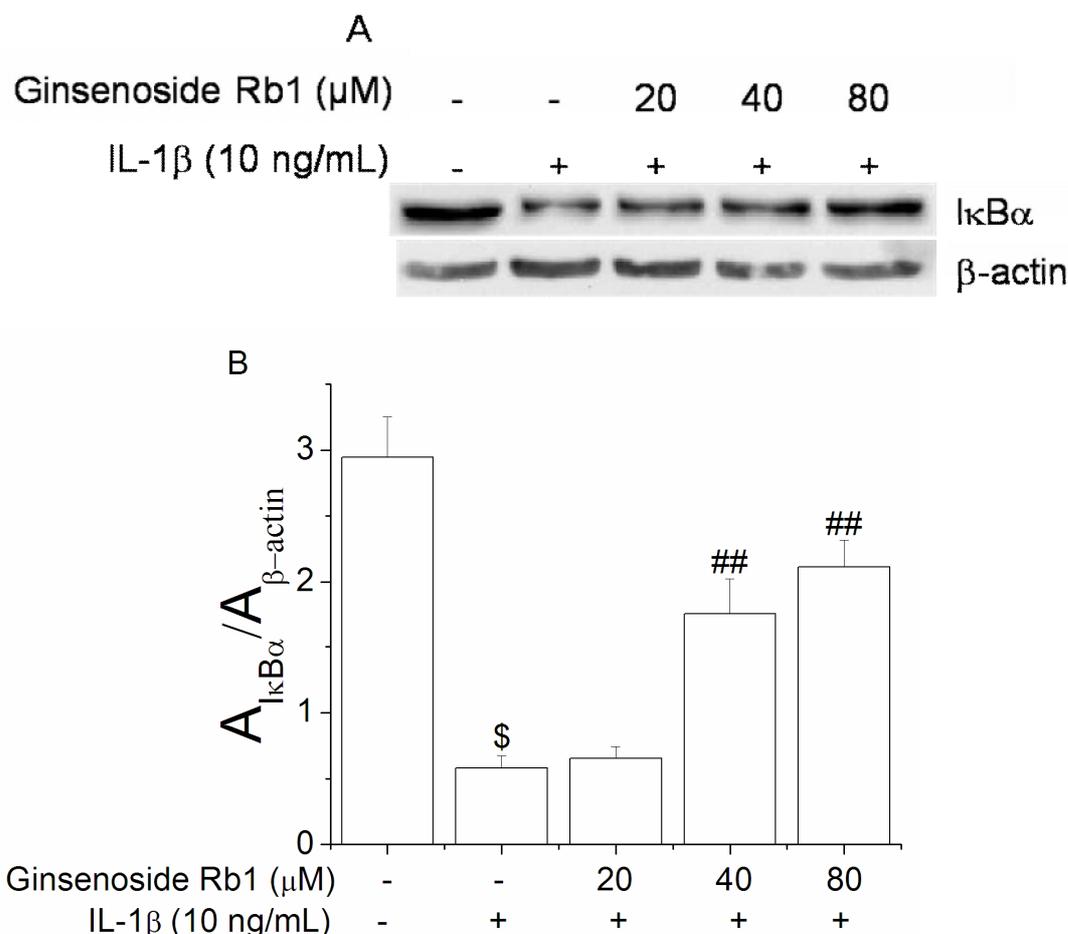


Figure 3: Effect of ginsenoside Rb1 on degradation of I κ B α in IL-1 β -induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μM) for 2 h and stimulated with 10 ng/mL IL-1 β for 30 min. (A) I κ B α protein in the cell lysates was detected by Western blot; (B) Bar graphs showed quantitative evaluation of I κ B α bands by densitometry. Three independent experiments performed in duplicate. Values are mean \pm SD; ^{\$} $p < 0.01$ vs. media alone-treated group; ^{##} $p < 0.01$ vs. IL-1 β alone-treated group

Effect of ginsenoside Rb1 on nuclear translocation of NF- κ B p65

To further determine whether ginsenoside Rb1 could modulate nuclear translocation of NF- κ B, nuclear level of NF- κ B p65 protein were examined in IL-1 β -stimulated SW1353 cells. Amounts of nuclear NF- κ B p65 were markedly increased after stimulation with IL-1 β alone for 1 h ($p < 0.01$, Fig 4A, B). Ginsenoside Rb1 reduced amounts of nuclear NF- κ B p65 in IL-1 β -stimulated SW1353 cells in a concentration-dependent manner, corresponding to approximate 30 % inhibition at 40 μM and 40 % at 80 μM (both $p < 0.01$, Fig 4B). However, 20 μM ginsenoside Rb1 did not significantly affect amounts of nuclear NF- κ B p65 in IL-1 β -stimulated SW1353 cells (Fig. 4A, B).

Effect of ginsenoside Rb1 on DNA binding activity of NF- κ B

Activation of NF- κ B is due to increased DNA binding after its dissociation from I κ B α . Since the p65 subunit has potent transcriptional activation domains, we investigated whether ginsenoside Rb1 could modulate DNA binding activity of NF- κ B p65 in IL-1 β -stimulated SW1353 cells in an ELISA-based assay. Upon exposure to IL-1 β alone, DNA binding activity of NF- κ B p65 was significantly increased within 1 h ($p < 0.01$, Fig. 5). Ginsenoside Rb1 decreased IL-1 β -induced DNA binding activity of NF- κ B p65 in a concentration-dependent manner, corresponding to approximately 40 % inhibition at 40 μM and 50 % at 80 μM (both $p < 0.01$, Fig. 5). Ginsenoside Rb1 at 20 μM did not have a significantly effect on IL-1 β -induced DNA binding activity of NF- κ B p65 (Fig. 5).

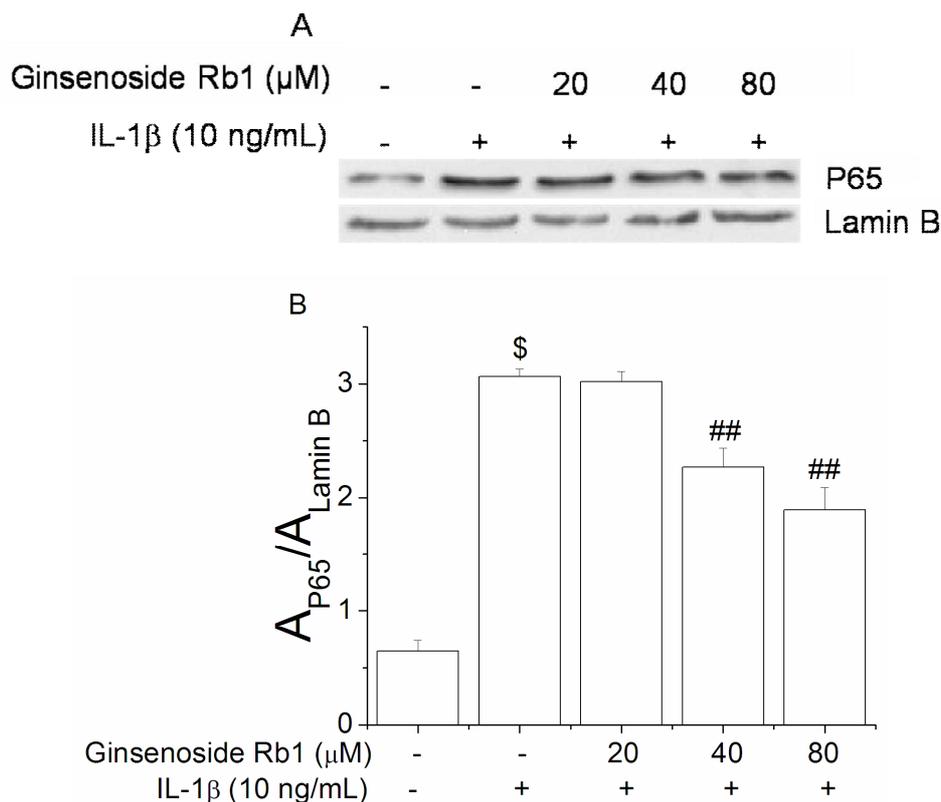


Figure 4: Effect of ginsenoside Rb1 on nuclear translocation of NF- κ B p65 in IL-1 β -induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μM) for 2 h and stimulated with 10 ng/mL IL-1 β for 1 h. (A) NF- κ B p65 protein in nucleus was detected by Western blot; (B) Bar graphs showed quantitative evaluation of NF- κ B p65 bands by densitometry. Three independent experiments performed in duplicate. Values are mean \pm SD; ^{\$} $p < 0.01$ vs. media alone-treated group; ^{##} $p < 0.01$ vs. IL-1 β alone-treated group

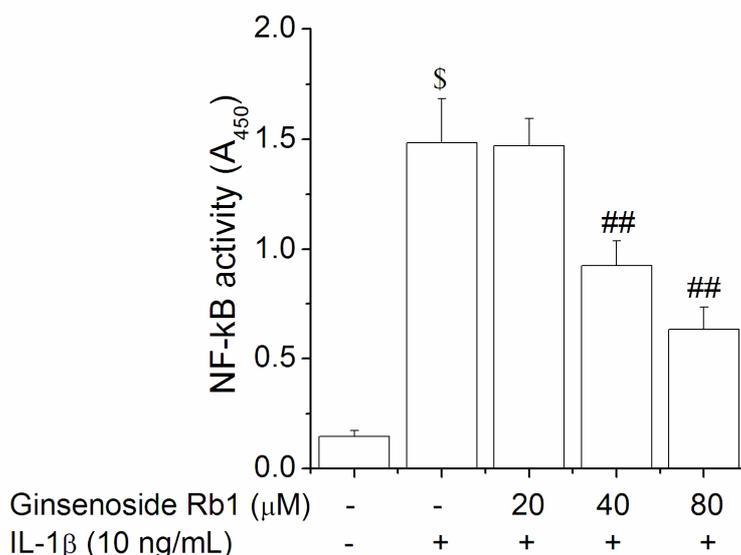


Figure 5: Effect of ginsenoside Rb1 on DNA binding activity of NF- κ B in IL-1 β -induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μM) for 2 h and stimulated with 10 ng/mL IL-1 β for 1 h. Nuclear protein extracts were used to measure DNA binding activity of NF- κ B complex with the Trans AM™ kit for p65. Three independent experiments performed in duplicate; values are mean \pm SD; ^{\$} $p < 0.01$ vs. media alone-treated group; ^{##} $p < 0.01$ vs. IL-1 β alone-treated group

DISCUSSION

In the present study, we demonstrated that ginsenoside Rb1, an active constituent in ginseng, could inhibit NO production in IL-1 β -induced SW1353 chondrocytes in a concentration-dependent manner. Meanwhile, concentrations of ginsenoside Rb1 used in this study were not cytotoxic to SW1353 cells, suggesting that this result was not due to reduced cell viability. Interestingly, evidence has verified that ginsenoside Rb1 can increase NO production in endothelial cells. Ginsenoside Rb1 prevented homocysteine-induced endothelial dysfunction by upregulating endothelial nitric oxide synthase (eNOS)/NO production in human umbilical vein endothelial cells [15]. It was also shown to abate homocysteine-induced endothelial dysfunction by increasing NO production and eNOS phosphorylation via PI3K/Akt activation and PKC inhibition [16]. Ginsenoside Rb1 had protective effects on oxLDL-injuring human vascular endothelial cells by increasing NO production and eNOS mRNA expressions [14]. Although these results appear to contradict our results, it is possible that regulation of NOS/NO signal by ginsenoside Rb1 varies in different pathological conditions.

Three isoforms of NOS have been identified, including inducible (iNOS), endothelial (eNOS) or neuronal NOS (nNOS) [17]. While the latter two are constitutively expressed, iNOS is expressed following stimulation with a variety of inflammatory agents such as endotoxins (LPS) or cytokines including IL-1 β and TNF- α . Under inflammatory conditions, iNOS is the key enzyme responsible for NO production [18]. Since we demonstrated the inhibitory effect of ginsenoside Rb1 on NO generation, we subsequently investigated the effect of ginsenoside Rb1 on iNOS protein expression in IL-1 β -induced SW1353 cells. Consistent with the inhibitory effect on NO production, ginsenoside Rb1 was found to attenuate IL-1 β -induced iNOS protein expression in SW1353 cells in a concentration-dependent manner. Especially, the inhibitory rate of ginsenoside Rb1 at 80 μ M exceeded 50 %. This data indicated that regulation of iNOS/NO signal in chondrocytes was the principle mechanism of anti-inflammation by ginsenoside Rb1.

NF- κ B activation has been implicated as a major mechanism for iNOS expression in IL-1 β -induced chondrocytes [19]. NF- κ B activation requires three key steps including degradation of I κ B α , nuclear translocation of NF- κ B p65 and DNA binding of the NF- κ B complex [20]. Numerous anti-inflammatory agents have been found to

regulate NF- κ B activation by interfering with one or more of these steps [21-24]. In this study, we found that ginsenoside Rb1 inhibited I κ B α degradation, as well as decreased levels of p65 protein in the nucleus of IL-1 β -induced SW1353 cells. These data suggested that ginsenoside Rb1 arrests the IL-1 β -induced nuclear translocation of NF- κ B p65. We also found that ginsenoside Rb1 inhibited the DNA binding activity of NF- κ B complex. These results indicated that ginsenoside Rb1 can inhibit NF- κ B activation through multiple mechanisms.

It has a long history of use in TCM to alleviate symptoms and delay the pathological development of OA, and ginseng is one of the most popular Chinese materia medica for OA treatment in TCM in China [25]. However, the mechanism by which the active components of ginseng exert their effects on OA are not clear. Our results show that ginsenoside Rb1 can inhibit NO production in IL-1 β -induced chondrocytes by its downregulating iNOS protein expression. This effect is attributed to the repression of NF- κ B by stabilizing I κ B α degradation, inhibiting nuclear translocation of p65 and impairing DNA binding activity of NF- κ B complex. Together our findings may in part explain the mechanisms by which ginseng exerts its beneficial effects in OA.

CONCLUSION

Ginsenoside Rb1 can significantly decrease NO production and iNOS protein expression in IL-1 β -induced SW1353 cells. This is accomplished through repression of NF- κ B activation via multiple mechanisms. These results suggest that ginsenoside Rb1 is a potential anti-NO agent for OA treatment, and reveals the underlying mechanism of the beneficial effect of ginseng in OA treatment in TCM. Subsequent studies are necessary to examine the effect of ginsenoside Rb1 in animal models of OA.

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