

Full Length Research Paper

Ginsenoside retinoblastoma 1 (Rb1) suppresses NO production and inducible nitric oxide synthase (iNOS) expression by inhibiting nuclear factor κ B (NF- κ B) activation in SW1353 chondrosarcoma cells

Ping Jia¹, Gang Chen², Rong-Heng Li^{1*}, Xiao-Feng Rong¹, Guo-Qing Zhou¹ and Yu Zhong¹

¹Department of Combination of Chinese and Western Medicine, the First Affiliated Hospital of Chongqing University of Medical Sciences, Chongqing 400016, P. R. China.

²Research Center of Medical Chemistry and Chemical Biology, Chongqing Technology and Business University, Chongqing 400067, P. R. China.

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Ginseng (*Panax ginseng* C.A. Mey) is commonly used to treat osteoarthritis (OA) in Chinese traditional medicine (TCM). In this study, we investigated whether ginsenoside retinoblastoma 1 (Rb1), an active component of ginseng, could regulate NO production in chondrocytes and its potential mechanisms of action. SW1353 cells were stimulated with IL-1 β in the presence of different concentrations of ginsenoside Rb1. NO concentration was assessed by the Griess reaction. Expression of iNOS, degradation of I κ B α and nuclear translocation of NF- κ B p65 were determined by Western blot. DNA binding activity of NF- κ B complex was evaluated with Trans AM™ kit for p65. We found that ginsenoside Rb1 significantly decreased the NO production and iNOS protein expression in a concentration-dependent manner. Ginsenoside Rb1 markedly decreased the I κ B α degradation and nuclear p65 levels, as well as inhibited the DNA binding activity of NF- κ B complex. 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 reveals potential mechanisms explaining the benefits of ginseng for OA treatment in TCM.

Key words: Ginsenoside, retinoblastoma 1 (Rb1), NO, inducible nitric oxide synthase (iNOS), nuclear factor κ B (NF- κ B), chondrocyte, osteoarthritis.

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 (Li, 2012). A growing body of evidence supports the fact that nitric oxide (NO) plays an important role in the pathological development of OA (Vuolteenaho et al., 2007). Osteoarthritic joints exhibit elevated NO production as well as increased amounts of other inflammatory media-

tors (Charles et al., 1993). Patients with osteoarthritis exhibit markers of enhanced NO production in their urine, serum, and synovial fluid (Spreng et al., 2001). In inflammatory reactions, NO is generated mainly by inducible nitric oxide 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 (Grabowski et al., 1997). NO is considered as a pro-inflammation agent as well as a

*Corresponding author. E-mail: rhli_tcm@yahoo.com.cn. Tel/Fax: (+86) 23-8901-2864.

potent catabolic mediator in OA since it promotes the production of inflammatory cytokines (Wang et al., 1997), activates matrix metalloproteinases (Murrell et al., 1995) and inhibits the synthesis of collagen and proteoglycan (Cao et al., 1997).

Nuclear factor κ B (NF- κ B) is a key transcription factor regulating iNOS gene expression in inflammatory conditions (Brown et al., 2008). 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) (Pasparakis, 2009). Concurrently, IL-1 β and TNF- α also function as potent inducers for NF- κ B activation.

Principal treatments for OA are mainly palliative for symptoms of dyskinesia and joint pain (Hunter, 2011). The use of ginseng dates back more than 2000 years in China, and is one of the most popular Chinese materia medica in TCM formulas for OA treatment (Cao et al., 2011). Ginsenoside retinoblastoma 1 (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 through inactivation of NF- κ B in lipopolysaccharide (LPS) -stimulated murine peritoneal macrophages (Joh et al., 2011). Although release of NO by ginsenoside Rb1 may underlie the cardiovascular protection (He et al., 2007), the effects and mechanism 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.

MATERIALS AND METHODS

Chemicals and reagents

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). dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St Louis, MO, 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 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 \times 10⁵ 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 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 measurements

NO production 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 RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) and EDTA-free protease inhibitor cocktail (Roche). To separate the nuclear proteins, cells were suspended in 250 μ l of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM 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

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-PAGE, and then transferred onto PVDF membranes. After blocking with 5% nonfat milk in TBST buffer (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 2 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. Chemiluminescent signals were detected and analyzed using the ChemiDoc XRS imaging system (Bio-Rad, USA).

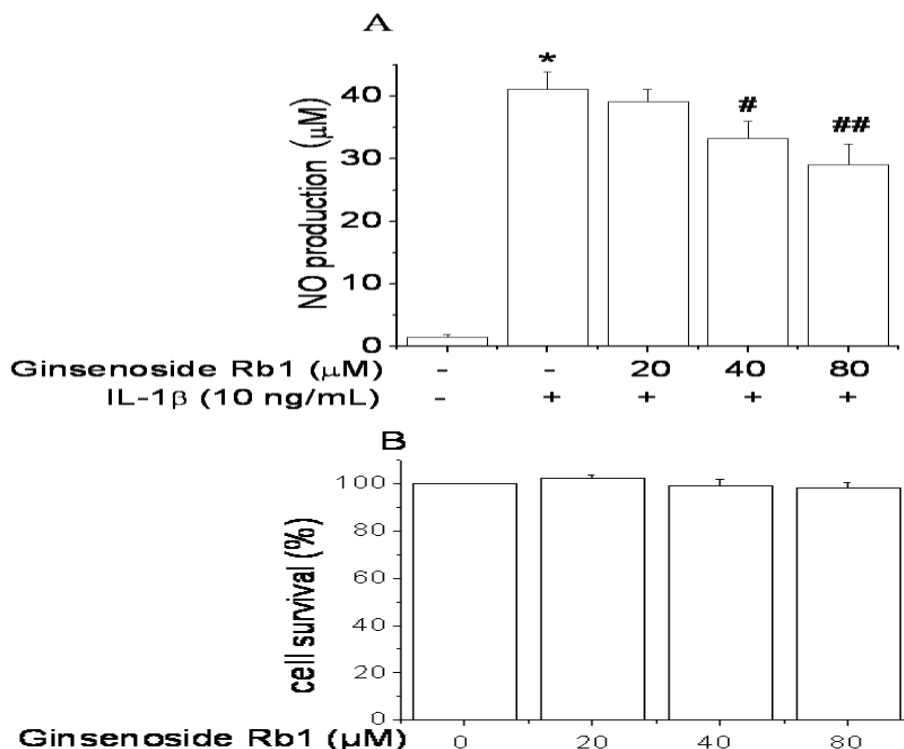


Figure 1. Effect of ginsenoside Rb1 on NO production in IL-1 β -induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μ M) for 2 h and stimulated with 10 ng/ml IL-1 β for 24 h. Secreted NO in the cell-free culture media was measured by Greiss reaction (A). SW1353 cells were incubated with ginsenoside Rb1 at different concentrations (20, 40, 80 μ M) for 24 h. MTT assay was performed to evaluate cytotoxicity (B). Three independent experiments performed in duplicate. Values are mean \pm SD. *, $p < 0.001$ vs. media alone-treated group. #, $p < 0.05$ or ##, $p < 0.01$ vs. IL-1 β alone-treated group.

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. 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 the Dunnett test. The differences were considered significant when $p < 0.05$.

RESULTS

Ginsenoside Rb1 inhibited NO production in IL-1 β -induced SW1353 cells

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 ($p < 0.001$) in the presence of IL-1 β after 24 h compared to baseline levels (Figure 1A). This effect was abrogated in a dose-dependent manner when SW1353 cells were cocultured with various concentrations of ginsenoside Rb1 (20 to 80 μ M). Ginsenoside Rb1 at 40 and 80 μ M decreased IL-1 β -induced NO production by approximately 24% ($p < 0.05$) and 46% ($p < 0.01$) respectively, but had no effect at 20 μ M (Figure 1). Ginsenoside Rb1 at the concentrations used in these experiments did not appear to be cytotoxic to the SW1353 cells (Figure 1B).

Ginsenoside Rb1 inhibited iNOS expression in IL-1 β -induced SW1353 cells

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 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 (Figure 2).

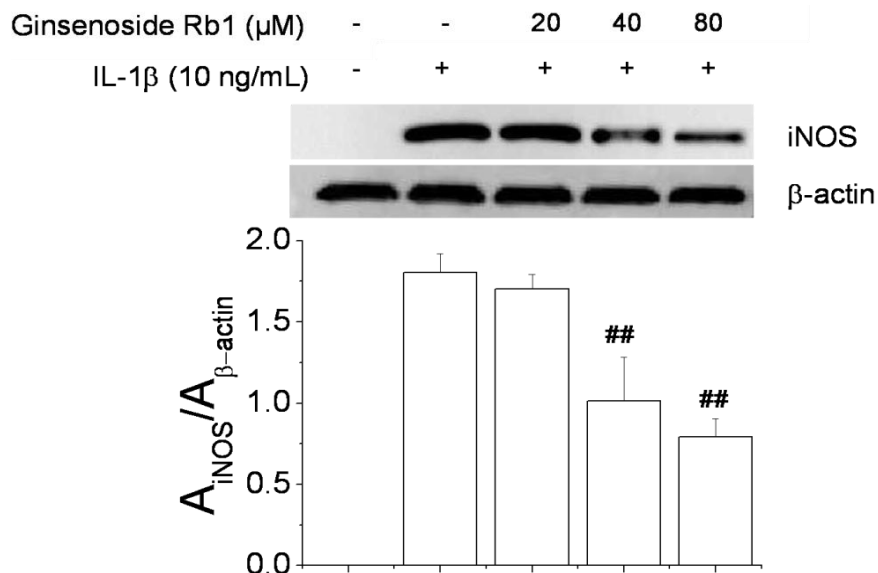


Figure 2. Effect of ginsenoside Rb1 on iNOS expression in IL-1 β -induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μM) for 2 h and stimulated with 10 ng/ml IL-1 β for 24 h. iNOS protein in the cell lysates was detected by Western blot. Three independent experiments were performed in duplicate. Values are mean \pm SD. ##, $p < 0.01$ vs. IL-1 β alone-treated group.

Ginsenoside Rb1 decreased IL-1 β -induced expression of iNOS protein in a concentration-dependent manner, corresponding to about 40% inhibition at 40 μM , 55% at 80 μM (both $p < 0.01$, Figure 2). Levels of iNOS protein were not significantly affected at 20 μM ginsenoside Rb1 (Figure 2).

Ginsenoside Rb1 inhibited degradation of I κ B α in IL-1 β -induced SW1353 cells

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. I κ B α degradation dramatically occurred within 30 min upon exposure to IL-1 β alone ($p < 0.001$, Figure 3). Ginsenoside Rb1 dramatically inhibited IL-1 β -induced I κ B α degradation by approximately 40 and 55% at 40 μM and 80 μM doses, respectively (both $p < 0.01$, Figure 3). However, ginsenoside Rb1 at 20 μM did not have a significantly inhibitory effect on IL-1 β -induced I κ B α degradation (Figure 3).

Ginsenoside Rb1 inhibited nuclear translocation of NF- κ B p65 in IL-1 β -induced SW1353 cells

To further determine whether ginsenoside Rb1 could modulate nuclear translocation of NF- κ B, nuclear extracts were examined from 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 ($p < 0.001$, Figure

4). Ginsenoside Rb1 inhibited IL-1 β -induced nuclear translocation of NF- κ B p65 in a concentration-dependent manner, corresponding to approximately 30% inhibition at 40 μM and 40% at 80 μM (both $p < 0.01$, Figure 4). However, 20 μM ginsenoside Rb1 did not significantly affect IL-1 β -induced nuclear translocation of NF- κ B p65 (Figure 4).

Ginsenoside Rb1 inhibited DNA binding activity of NF- κ B complex in IL-1 β -induced SW1353 cells

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 -induced 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 ($p < 0.001$, Figure 5). Ginsenoside Rb1 decreased IL-1 β -induced DNA binding activity of NF- κ B p65 in a concentration-dependent manner (Figure 5), corresponding to approximately 40% inhibition at 40 μM and 50% at 80 μM ($p < 0.01$, Figure 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 (Figure 5).

DISCUSSION

In the present study, we found that ginsenoside Rb1, the main active constituent in ginseng, could inhibit NO

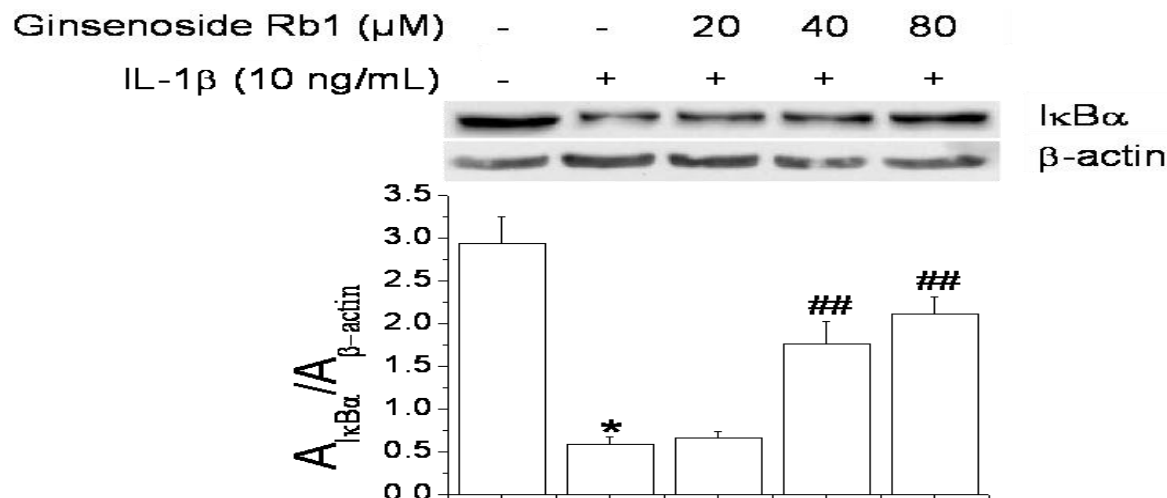


Figure 3. Effect of ginsenoside Rb1 on degradation of I κ B α in IL-1 β -induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μM) for 2 h and stimulated with 10 ng/ml IL-1 β for 30 min. I κ B α protein in the cell lysates was detected by Western blot. Three independent experiments performed in duplicate. Values are mean \pm SD. *, $p < 0.001$ vs. media alone-treated group. ##, $p < 0.01$ vs. IL-1 β alone-treated group.

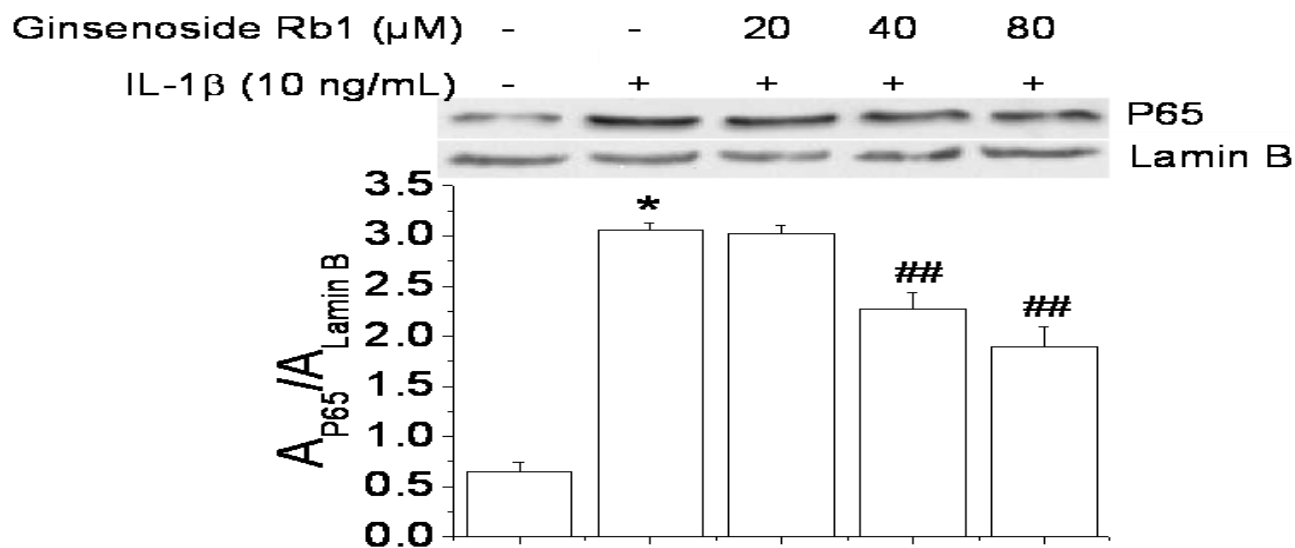


Figure 4. Effect of ginsenoside Rb1 on nuclear translocation of NF- κ B p65 in IL-1 β -induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μM) for 2 h and stimulated with 10 ng/ml IL-1 β for 1 h. NF- κ B p65 protein in nucleus was detected by Western blot. Three independent experiments performed in duplicate. Values are mean \pm SD. *, $p < 0.001$ vs. media alone-treated group. ##, $p < 0.01$ vs. IL-1 β alone-treated group.

production in IL-1 β -induced SW1353 chondrocytes in a concentration-dependent manner. Concentrations of ginsenoside Rb1 used in this study were not cytotoxic to SW1353 cells thus these results were not due to reduced cell viability. Evidence suggests that ginsenoside Rb1 can increase NO production in endothelial cells. Ginsenoside Rb1 was previously found to prevent homocysteine-induced endothelial dysfunction by upregulating endothelial nitric oxide synthase (eNOS)/NO production in human umbilical vein endothelial cells (Xu et al., 2011).

It was also shown to abate homocysteine-induced endothelial dysfunction by increasing NO production and eNOS phosphorylation via PI3K/Akt activation and PKC inhibition (Lan et al., 2011). Ginsenoside Rb1 had protective effects on oxLDL-injuring human vascular endothelial cells by increasing NO production and eNOS mRNA expressions (He et al., 2007). Although these reports appear to contradict our results, it is possible that regulation of NOS/NO signal by ginsenoside Rb1 varies in different pathological conditions.

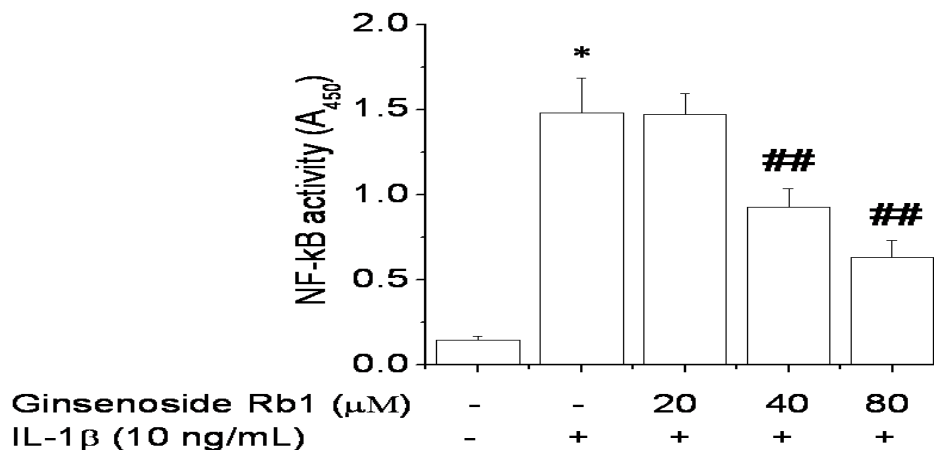


Figure 5. Effect of ginsenoside Rb1 on DNA binding activity of NF-κB complex in IL-1β-induced SW1353 cells. SW1353 cells were pre-treated with ginsenoside Rb1 (20, 40, 80 μM) for 2 h and stimulated with 10 ng/ml IL-1β for 1 h. Nuclear protein extracts were used to measure DNA binding activity of NF-κB complex with the Trans AM™ kit for p65. Three independent experiments were performed in duplicate. Values are mean ± SD. *, p<0.001 vs. media alone-treated group. **, p<0.01 vs. IL-1β alone-treated group.

Three isoforms of NOS have been identified, including inducible (iNOS), endothelial (eNOS) or neuronal NOS (nNOS) (Kobayashi, 2010). 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-1β and TNF-α. Under inflammatory conditions, iNOS is the key enzyme responsible for NO production (Abramson et al., 2001). Therefore we investigated the inhibitory effect of ginsenoside Rb1 on iNOS expression in IL-1β-induced SW1353 cells. Consistent with the inhibitory effect on NO production, ginsenoside Rb1 was found to attenuate IL-1β-induced iNOS protein expression in SW1353 cells.

NF-κB activation has been implicated as a major mechanism for iNOS expression in IL-1β-induced chondrocytes. NF-κB activation requires three key steps including degradation of IκBα, nuclear translocation of NF-κB p65 and DNA binding of the NF-κB complex (Renner and Schmitz, 2011). Numerous anti-inflammatory agents have been found to regulate NF-κB activation by interfering with one or more these processes (Sethi and Tergaonkar, 2009). Chondroitin sulfate, a glucosaminogly can now be used for the treatment of OA in clinical practice, inhibited the nuclear translocation of NF-κB in IL-1β-stimulated chondrocytes (Jomphe et al., 2008) and in LPS-stimulated rat astrocytes (Cañas et al., 2010). N¹-Benzyl-4-methylbenzene-1,2-diamine, a novel synthetic compound, inhibited nuclear translocation of NF-κB p65 and DNA binding activity of NF-κB complex in parallel, but did not affect the degradation of IκBα in LPS-stimulated RAW 264.7 macrophages (Shin et al., 2005). Ethyl caffeate, a natural phenolic compound isolated from *Bidens pilosa*, did not show inhibitory effect on the phosphorylation and degradation of IκBα and the translocation of NF-κB into the nucleus in LPS-induced

RAW 264.7 macrophages. However, ethyl caffeate could inhibit NF-κB activation by impairing the NF-κB DNA binding (Chiang et al., 2005). In this study, we found that ginsenoside Rb1 inhibited IκBα degradation, as well as decreased levels of p65 protein in the nucleus of IL-1β-induced SW1353 cells. This indicated that ginsenoside Rb1 arrests the IL-1β-induced nuclear translocation of NF-κB p65. We also found that ginsenoside Rb1 inhibited the DNA binding activity of NF-κB complex. These results indicated that ginsenoside Rb1 can inhibit NF-κB activation through multiple mechanisms.

The use TCM to alleviate symptoms and delay the pathological development of OA has a long history with ginseng among the most popular in Chinese materia medica. However, the mechanisms 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-1β-induced chondrocytes by its down regulating iNOS protein expression. This effect was attributed to the repression of NF-κB by stabilizing IκBα degradation, inhibiting nuclear translocation of p65 and impairing DNA binding activity of NF-κB complex. Together our findings may in part explain the mechanisms by which ginseng exerts its beneficial effects in OA.

Conclusion

It is clear that ginsenoside Rb1 can significantly decrease the NO production and iNOS protein expression in IL-1β-induced SW1353 cells. This was accomplished through repression of NF-κB activation through multiple mechanisms. Subsequent studies are necessary to examine the effects of ginsenoside Rb1 in animal models

of osteoarthritis, and further study the pharmacology as well as side effects of ginsenoside Rb1.

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