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Full Length Research Paper

Salvianolic acid B inhibits matrix metalloproteinases by NF-κB signaling in rabbit chondrocytes

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This study investigates the chondroprotective properties of Salvianolic acid B (Sal B) in interleukin (IL)-1β-induced rabbit chondrocytes. Quantitative real-time PCR, enzyme-linked immunosorbent assay (ELISA) and western blot were performed to investigate the effects of Sal B on the mRAN and protein levels of matrix metalloproteinases (MMP)-1, MMP-3, MMP-13, tissue inhibitors of metalloproteinase-1 (TIMP-1) and collagen II in IL-1β-induced rabbit chondrocytes. Sal B inhibits the mRNA level as well as the protein level of these MMPs. Sal B also increases IL-1β-inhibited mRNA and protein levels of TIMP-1 and collagen II. In addition, Sal B inhibits the IL-1β-induced activation of nuclear factor kappa B (NF-κB) and the degradation of inhibitor of κB (IκB)-α. Our data suggest that Sal B exhibit chondroprotective properties in chondrocytes.

Key words: Salvianolic acid B, osteoarthritis, matrix metalloproteinase, chondrocyte, interleukin-1 beta, NF-κB.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease in which many risk factors are implicated. For instance, matrix metalloproteinases (MMPs), a group of matrix-degrading enzymes, contribute to cartilage degradation via cleaving cartilage matrix such as proteoglycan and collagens (Burrage et al., 2006). The activity of MMPs is antagonized by tissue inhibitors of metalloproteinases (TIMPs) which are specific inhibitors of MMPs (Martel-Pelletier et al., 2001). Excess of MMPs over TIMPs in arthritic-cartilage has been documented (Martel-Pelletier et al., 1994). In addition, the pro-inflammatory cytokine interleukin-1 beta (IL-1β) also plays an important role in the progression of OA (Pelletier et al., 1991). IL-1β participates in the pathogenesis of OA by up-regulating the levels of MMPs (Tetlow et al., 2001).

The over-expression of MMPs and pro-inflammatory cytokines is related to the activation of nuclear factor-kappa B (NF-κB), an important transcription factor involved in arthritis (Liacini et al., 2003; Lianxu et al., 2006). Induction of MMPs by activation of NF-κB in chondrocytes has been reported by Liacini et al. (2002). Several studies demonstrated that agents which inhibited the activation of NF-κB showed beneficial effects in arthritis (Ahmed et al., 2004; Lauder et al., 2007). Furthermore, NF-κB inhibitors have been shown to exert beneficial effects in the treatment OA in animals (Chen et al., 2008). Thus, there is a great interest to consider NF-κB as a potential therapeutic target in the treatment of OA (Roman-Blas and Jimenez, 2006).

Salvianolic acid B (Sal B) is a water-soluble compound extracted from *Salvia miltiorrhiza*. Sal B is well known for its beneficial effects on cardiovascular diseases (Zhou et al., 2005). *In vitro* and *in vivo* studies documented the biological action of Sal B (Zhao et al., 2008; Chen et al., 2009; Lin et al., 2006). Furthermore, several studies

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Abbreviations: OA, Osteoarthritis; **ECM**, extracellular matrix; MMPs, matrix metalloproteinases; **TIMPs,** tissue inhibitors of metalloproteinases; **IL-1β**, interleukin-1 beta; **MTT,** 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-kappa **B; Sal B**, salvianolic acid **B; PCR,** polymerase chain reaction]

Table 1. Primers of targeted genes.

Targeted gene	Sequence(5'-3')*	Amplicon length (bp)	Accession number
Collagen II	S: GACTGCCTGAGCCCCGAGAT A: CTGCCCCTTTGGTCCTGGTTTC	95	D83229
MMP-1	S: GACGTGGACCGACAACAGTGA A: GGGGAACATTAGTGCTCCTACATC	112	AH005676
MMP-3	S: ACACCGGATCTGCCAAGAGA A: CTGGAGAACGTGAGTGGAGTCA	89	NM001082280
MMP-13	S: TTGACCACTCCAAGGACCCAG A: GAGGATGCA GACGCCAGAAGA	252	NM001082037
TIMP-1	S: CAACTGCGGAACGGGCTCTTG A: CGGCAGCGTAGGTCTTGGTGAA	102	AY8297305
18S rRNA	S: GACGGACCAGAGCGAAAGC A: CGCCAGTCGGCATCGTTTATG	119	EU236696

*, S = Sense; A = antisense.

demonstrated that Sal B can inhibit NF-κB activation in various cells (Zhou et al., 2005). These findings indicate that Sal B may be beneficial to the treatment of OA. In this study, we investigated whether Sal B exerted chondroprotective effects in rabbit chondrocytes.

MATERIALS AND METHODS

Reagents

Sal B was obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), IL-1β and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin, fetal bovine serum (FBS), 0.25% trypsin and collagenase II were obtain from Gibco BRL (Grand Island, NY). Mouse monoclonal anti-Collagen II and goat anti-mouse antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The study was approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China). 5-week-old female China white rabbits (Animal Center, Zhejiang University) were used for chondrocytes culture. Cells were isolated from cartilage as described previously_(Baek et al., 2006). Cells were cultured in DMEM with 10% FBS and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin) at 37°C with 5% CO_2. Confluent primary chondrocytes were split at a ratio of 1:3. Second passages of chondrocytes were used in the present study.

Cell viability assay

Chondrocytes were cultured at a density of 6×10^3 /well in 96-well plates and treated with various concentrations of Sal B followed for 24 h. MTT was used in cell viability assays. This involved adding 20 µl of MTT/well and incubating the cells for 4 h. After aspiration of the supernatant, dimethyl sulfoxide (DMSO) were added, 150 µl/well. The plate was shaken for 10 min. Absorbance at 570 nm was measured with a micro-plate reader (Bio-Rad, Hercules, CA, USA). Medium without serum and cells were used as the zero point of absorbance.

Quantitative real-time PCR

Chondrocytes were transferred to six-well plates at the density of 10^5 cells/well. Sub-confluent cells were serum-starved overnight. Sal B was added 1 h before the stimulation with 10 ng/ml of IL-1β for 24 h. RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Reverse transcription of RNA was accomplished as described previously (Bao et al., 2009). Quantitative real-time PCR was performed using iCycler apparatus system (Bio-Rad, Hercules, CA, USA) and SYBR green detection technology. The sequences of primers used are shown in Table 1. 18S rRNA was used as an endogenous control. The cycle threshold (CT) values for each gene were corrected using the mean CT value. Real-time PCR data were quantified using the ΔCT method with the formula: $n = 100 \times 2^{-(_{\Delta}CT\ \text{targeted gene}-_{\Delta}CT\ 18s\ rRNA)}$.

Enzyme-linked immunosorbent assay (ELISA)

Confluent cells were serum-starved overnight and treated with Sal B 1 h prior to IL-1β (10 ng/ml) for 24 h. Culture medium was collected for ELISA assay using rabbit MMP-1, MMP-3, MMP-13

and TIMP-1 ELISA kits according to the manufacturer's instruction (R&D Systems, Inc., Minneapolis, MN, USA).

Western blot analysis

Chondrocytes were pretreated with or without Sal B for 24 h, then stimulated with IL-1β (10 ng/ml) for 1 h. Cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS). A protein extraction kit was used for nuclear and cytoplasmic proteins extraction (Beyotime Institute of Biotechnology, Jiangshu, China) and the protein concentration was quantified using Bradford assay. Protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were pre-incubated with blocking buffer (5% (w/v) skimmed milk powder in Tris-buffered saline and 0.1%Tween-20) for 1 h and were incubated with primary antibodies against collagen II, NF-κB p65, Lamin B, IκB-α and β-actin (1:1000) for overnight at 4°C (Santa Cruz Biotechnology, CA, USA). Membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase (HRP) -linked secondary antibodies (1:1,000). After washing, membranes were detected by Enhanced Chemiluminescence (ECL) kit and exposed to X-ray films (Kodak, Hanzhou,China).

Immunofluorescence staining

Chondrocytes in slides were serum-starved overnight, then, cells were pretreated with Sal B for 24 h and stimulated with 10 ng/ml of IL-1β for 1 h, fixed with 4% paraformaldehyde, washed with PBS, blocked with 5% goat serum for 30 min, incubated with rabbit polyclonal anti-NF-κB p65 antibody overnight and PE-conjugated goat anti-rabbit antibody for 60 min (Santa Cruz Biotechnology, CA, USA), counterstained with 4'-6-diamidino-2-phenylindole for 30 min, viewed on confocal microscopy (Carl Zeiss, Oberkochen,Germany).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis of the data was performed by a paired t test. Differences were considered significant at $p < 0.05$.

RESULTS

Effects of Sal B on mRMA level of MMP-1, MMP-3, MMP-13, TIMP-1 and collagen II in IL-1β-induced chondrocytes

IL-1β induced the mRNA level of MMP-1, MMP-3, MMP-13 and inhibited the mRNA level of TIMP-1 and collagen II. Sal B, at concentrations of 10 and 20 μM inhibited the mRNA level of MMP-1 MMP-3, and MMP-13 and increased the mRNA level of TIMP-1 and collagen II ($p < 0.05$) (Figure 1) without cytotoxicity (Figure 2).

Effect of Sal B on protein level of MMP-1, MMP-3, MMP-13, TIMP-1 and collagen II in IL-1β-stimulated chondrocytes

Stimulation with IL- 1β resulted in the increased protein level of MMP-1, MMP-3 and MMP-13 while the protein level of TIMP-1 and collagen II were decreased. Sal B at a concentration of 20 μM significantly down-regulated the protein level of MMP-1, MMP-3, MMP13, and up-regulated the protein level TIMP-1 and collagen II ($p < 0.05$) (Figure 3).

Sal B inhibited IκB-α degradation and NF-κB activation in chondrocytes

IL-1β induced the protein expression of NF-κB p65 and degraded the IκB-α. Sal B at a concentration of 20 μM significantly inhibited NF-κB p65 expression (Figure 4A) and the degradation of IκB-α (Figure 4B). The results were consistent with immunofluorescence staining showing that Sal B suppressed the NF-κB p65 staining in the nuclei (Figure 5).

DISCUSSION

In the present study, we found that Sal B, a compound extracted from *S. miltiorrhiza* showed anti-arthritic properties in rabbit chondrocytes, and these effects were associated with the inhibition of NF-κB activation.

MMPs have been known to induce extracellular matrix (ECM) degradation. MMPs, especially MMP-1 and MMP-13 are well known for the ability to degrade collagen II, the main component of ECM. Findings from studies using MMPs inhibitors in chondrocytes confirm the catabolic role of MMPs in OA (Piecha et al., 2010; Johnson et al., 2007). Dysregulation between MMPs and TIMPs are implicated in the development of OA. Thus, it is reasonable to consider that down-regulation of MMPs and up-regulation of TIMPs are beneficial to the treatment of OA. In the present study, IL-1β markedly induced the mRNA and protein level of MMP-1, MMP-3 and MMP-13, and decreased the mRNA and protein level of TIMP-1 in chondrocytes. The results are consistent with previous studies, confirming the catabolic role of IL-1β in arthritis (Phitak et al., 2009). We demonstrated that Sal B significantly inhibited the mRNA and protein level of MMP-1, MMP-3, MMP-13, and increased the mRNA and protein level of TIMP-1 and collagen II in IL-1β-induced rabbit chondrocytes. Our results indicate that Sal B affect the MMPs/TIMPs system in chondrocytes. The inhibitory effect on MMPs by Sal B has been reported by Liang et al. (2009) they demonstrated that Sal B inhibited the activities of MMP-1, MMP-2 and MMP-9 (Liang et al., 2009). Another study by Lin et al. (2007) demonstrated that Sal B attenuated MMP-2 and MMP-9 expression in human aortic smooth muscle cells followed by stimulation of lipopolysaccharide (LPS). All these results indicate that Sal B may exert inhibitory effects on MMPs in various cells.

In this study, we found that Sal B inhibited NF-κB

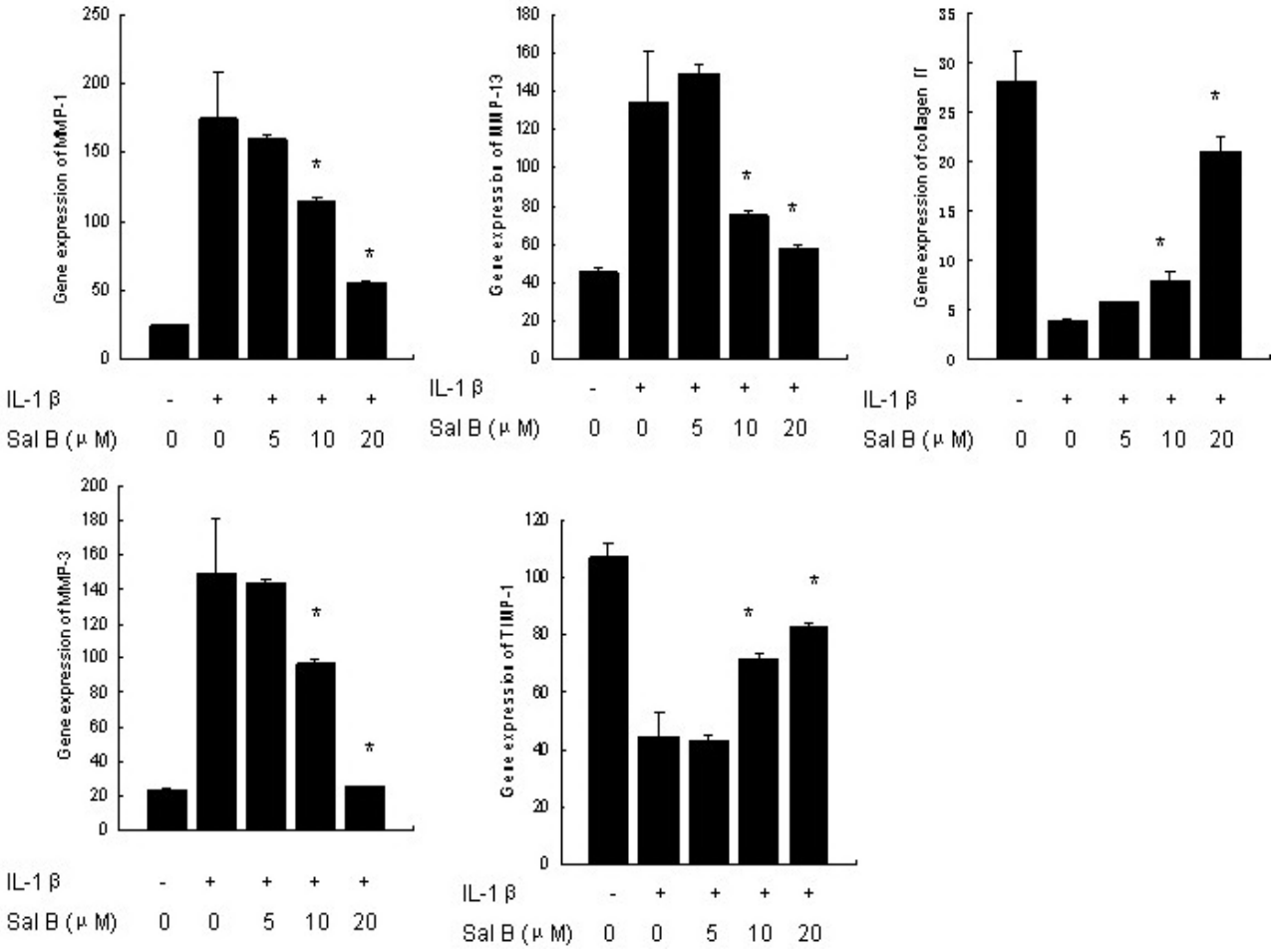

Figure 1. Effects of Sal B on mRNA level of MMP-1, MMP-3, MMP-13, TIMP-1 and collagen II in chondrocytes. Chondrocytes were treated with 10 ng/ ml of IL-1β in the absence or presence of Sal B for 24 h. The mRNA level were analyzed by quantitative real-time PCR. Results are expressed as mean ± standard deviation (SD). *, $p< 0.05$ compared with cells treated with IL-1β alone.

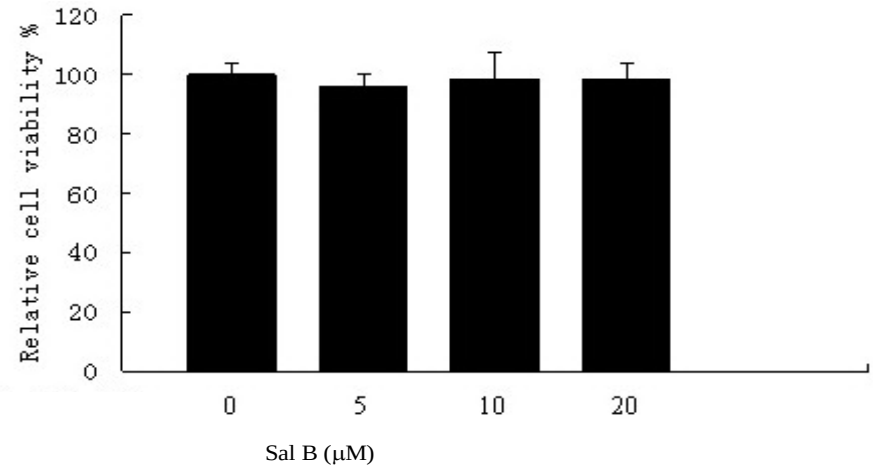

Figure 2. MTT assay for cell viability in chondrocytes. Chondrocytes were treated with various concentration of Sal B for 24 h. Cell viability was unaffected by Sal B as compared with normal cells.

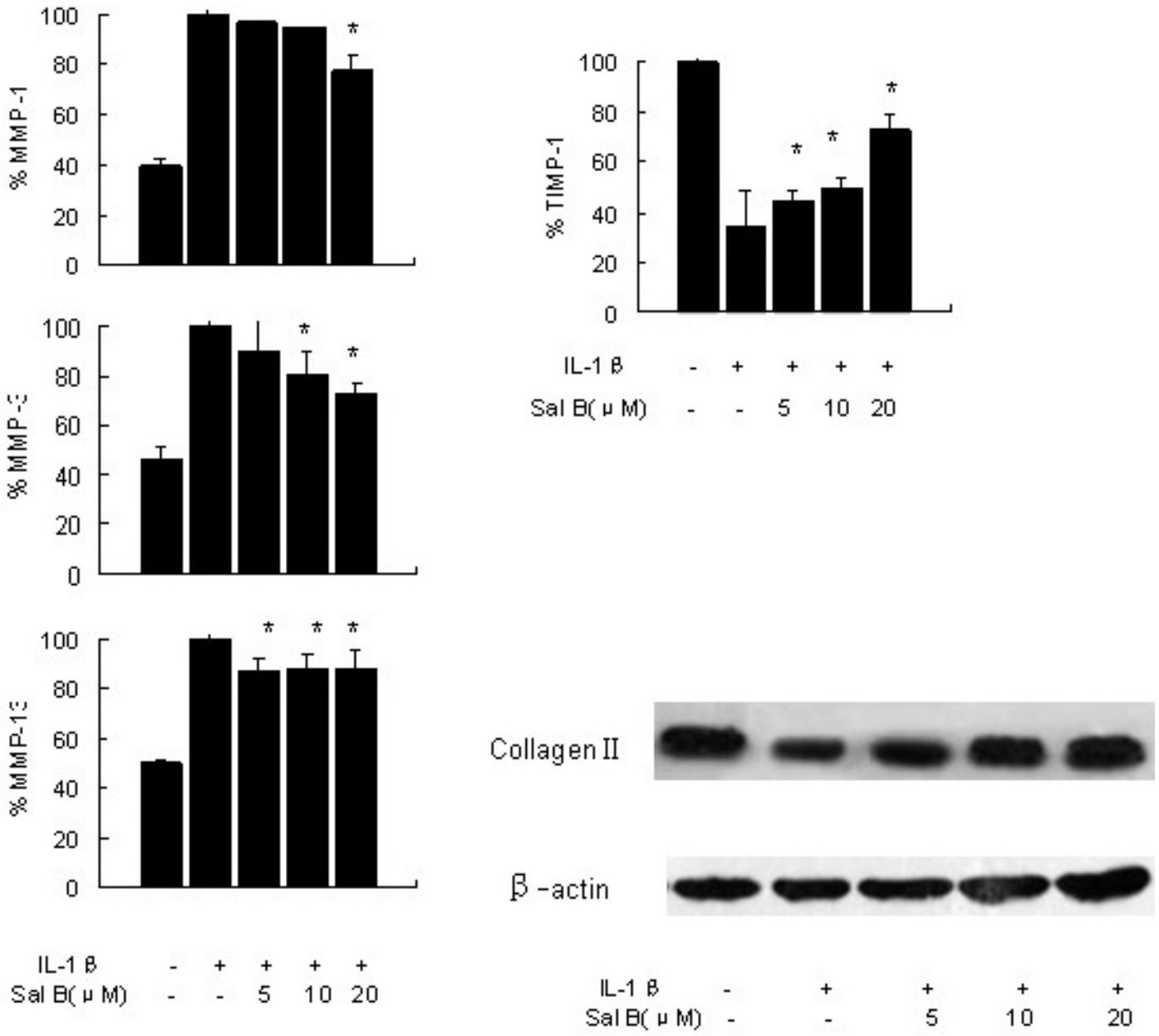

Figure 3. Effects of Sal B on the protein level of MMP-1, MMP-3, MMP-13, TIMP-1 and collagen II in chondrocytes. Chondrocytes were treated with Sal B 1 h prior to IL-1β (10 ng/ml) for 24 h. The protein level of MMP-1, MMP-3, MMP-13 and TIMP-1 were analyzed by ELISA, the protein level of collagen II was analyzed by western blot. *, $p < 0.05$ compared with cells treated with IL-1β alone.

activation as well as the degradation of IκB-α. NF-κB has. been shown to involved in the up-regulation of pro-inflammatory cytokines IL-1 and tumour necrosis factor alpha (TNF-α) as well as inflammatory factors such as cyclooxygenase (COX-2) and inducible NO synthase (iNOS) (Shalom-Barak et al., 1998; Tak and Firestein., 2001). All these mediators play important roles in the progression of OA. Furthermore, previous studies have demonstrated that NF-κB was associated with the MMPs up-regulation at mRNA or protein levels, leading to ECM degradation and cartilage damage (Liacini et al., 2003; Vincenti et al., 2002). Once activated, NF-κB p65 immigrates from cytoplasm to the nucleus to regulate gene expression. In this study, increased protein levels of nuclear NF-κB p65 in response to IL-1β was noted in chondrocytes. Our results are consistent with the finding of other studies showing that IL-1β can increase the expression of NF-κB in chondrocytes (Gabay et al., 2009; Sylvester et al., 2001; Domagala et al., 2006). Sal B has been shown to suppress the activation of NF-κB in various cells. For instance, Zhe et al. (2005) demonstrated that Sal B inhibited the TNF-α induced NF-

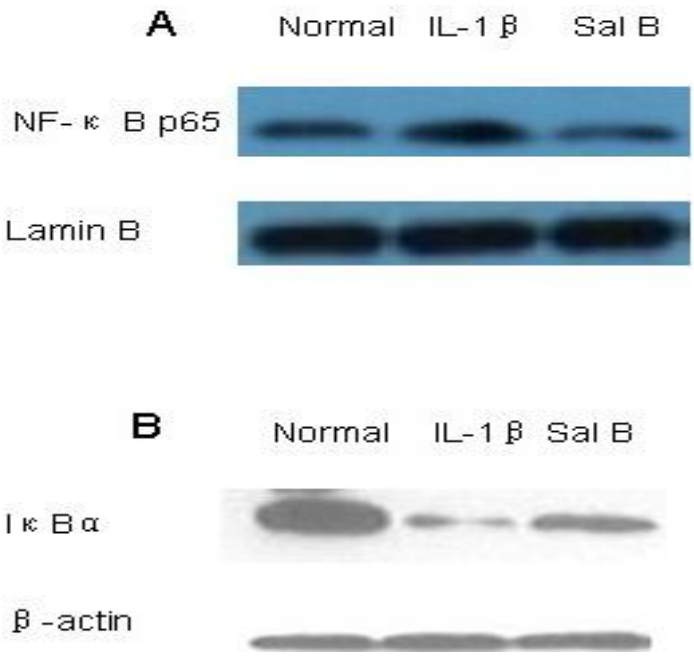

Figure 4. Sal B inhibited IκB-α degradation and NF-κB activation in chondrocytes. Chondrocyte were pre-cultured in serum-free medium in the presence or absence of Sal B(20 μM) for 24 h, and stimulated with IL-1β (10 ng/ml) for 1 h. Sal B at 20 μM significantly inhibited the protein level of NF-κB p65 (A) and the IκB-α degradation (B).

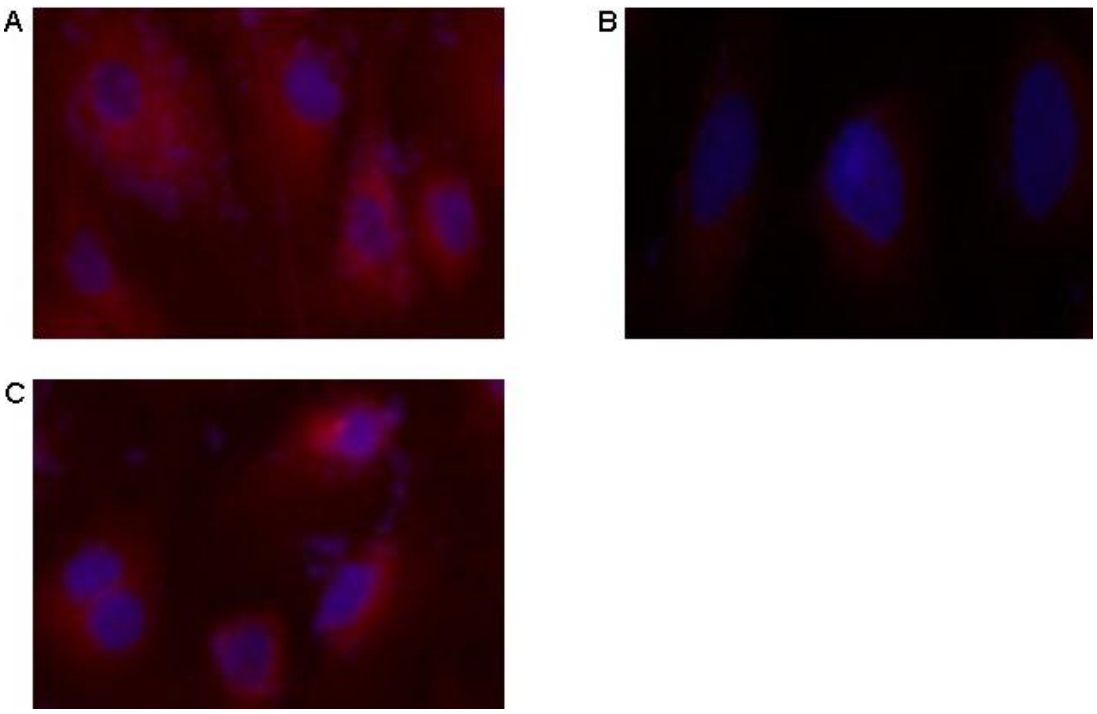

Figure 5. Immunofluorescence staining of NF-κB p65 in chondrocytes. Chondrocytes were pretreated with Sal B for 24 h and stimulated with 10 ng/ml of IL-1β for 1 h. The translocation of NF-κB p65 was investigated by immunofluorescence staining. A, Normal chondrocytes; B, IL-1β (10 ng/ml) treatment; C, Sal B (20 μM) + IL-1β (10 ng/ml) treatment.

κB activation in human umbilical vein endothelial cells. Luo et al. (2008) found that Sal B inhibited NF-κB activation in mesangial cells. Following these findings, it was interesting to investigate whether Sal B can exert its inhibitory effects on NF-κB activation in chondrocytes. We demonstrated that Sal B inhibited NF-κB activation in rabbit chondrocytes as well as the degradation of IκB-α. In addition, Sal B inhibited the translocation of NF-κB p65 which is a required step for NF-κB activation. Thus, we speculated that the inhibitory effects on MMPs by Sal B may be partly associated with the suppression of NF-κB activation. Further studies are needed to elucidate the exact mechanism by which Sal B regulates MMPs/TIMPs system.

In conclusion, this study demonstrated that Sal B exerted chondroprotective properties which may partly associate with the inhibitory effects on NF-κB. Our data suggest that Sal B may be considered as a potential therapeutic agent in the treatment of OA.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors declared that there are no conflicts of interest.

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