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Protective effect of kobophenol A on nitric oxide-induced cell apoptosis in human osteoblast-like MG-63 cells: Involvement of JNK, NF-κB and AP-1 pathways

Sung-Ryul Lee, Jong-Hwan Kwak, Dae-Sup Park, Suhkneung Pyo *

School of Pharmacy, Sungkyunkwan University, Suwon, Gyeonggi-do 440-746, Republic of Korea

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ABSTRACT

Nitric oxide (NO) is a multifunctional signaling molecule and the cytotoxic species responsible for a variety of pathologic disorders including bone destruction. High NO levels induce the apoptosis of osteoblasts and decrease the bone mineral density. We investigated the influence of kobophenol A (kob A) on apoptosis in cultured human osteoblast-like MG-63 cells. Direct NO donor sodium nitroprusside (SNP) that has been recognized as an inducer of apoptosis in various cell lines significantly induced cell death and NO production in MG-63 cells. Coincubation of kob A in SNP-treated MG-63 cells resulted in a significant protection against NO-induced cell death. This is associated with increase in intracellular reactive oxygen species (ROS) scavenging activity and the inhibition of decrease in mitochondrial membrane potential (MMP) by kob A. We also found that kob A inhibited the down-regulation of Bcl-2 and Bcl-X_L, whereas the level of Bax expression was decreased by kob A treatment in SNP-treated MG-63 cells. Furthermore, kob A inhibited SNP-induced phosphorylation of JNK and c-Jun, and SNP-induced reduction in NF-κB and AP-1 activities, implicating that protective effect of kob A may occur through the regulation of JNK, NF-κB and AP-1 signaling pathways. Together, these findings suggest that kob A has a protective effect against NO-mediated osteoblast apoptosis and might be a plausible candidate for treatment of inflammatory bone diseases relevant to osteoblast cell death.

1. Introduction

Bone continuously undergoes a process of renewal and repair through bone remodeling. Bone cell proliferation, bone formation, and bone resorption are the major factors involved in maintaining the bone mass. The concept of the bone cell life span in regulating the bone mass has emerged because apoptotic cell death plays an important role in the bone remodeling process [1,2]. Osteoblast apoptosis or programmed cell death has been observed in both humans and mice after estrogen deprivation [3,4]. Moreover, experimental and clinical studies have strongly suggested a link between the increased inflammatory cytokine activity including TNF-α and post-menopausal bone loss [5]. Estrogen exerts its biological function through estrogen receptor-mediated or its receptor-independent actions by changing the intracellular calcium, cAMP, and nitric oxide levels, as well as the activity of a variety of kinases [6]. In addition to estrogen deprivation, shear stress or other inflammatory cytokines, nitric oxide (NO) is associated with the apoptosis of osteoblasts [7–10]. However, the precise mechanisms underlying the association of osteocyte apoptosis with osteoporosis remain unclear.

NO is a highly reactive free radical that is involved in the regulation of many physiological processes, such as vascular relaxation, neurotransmission and platelet aggregation. Recently, it has become apparent that NO has important effects on the bone cell function [11]. Constitutive NO can be an effective mediator that regulates the proliferation and differentiation of osteoblasts [10]. In contrast, the overproduction of NO leads to osteoblast injury [8,9,12]. Thus, NO has both pro-apoptotic or anti-apoptotic effects in cells. In addition, NO potentiates the cytokine-induced bone resorption and high levels of NO can inhibit bone formation.

Caragana sinica (Buc's hoz) Rhed is widely distributed in Korea, Japan and China. The subterranean parts of *C. sinica* are rich in oligostilbenes, such as miyabenol C, (+)-α-viniferin, caraganaphenol A and kobophenol A (kob A), and the herb has been used in traditional medicine to treat neuralgia, hypertension and arthritis. Kob A, a tetrameric stilbene, is one of the major active compounds from *C. sinica* [13,14]. Kob A exhibits protein kinase C inhibitory activity, antimicrobial activity and neuroprotective effects from NO or mitochondrial damage [13–15]. Kob A is also known to be a phytoestrogen through an E-screen assay and has the capacity of binding to the estrogen receptor [16,17]. However, the mechanism by which kob A exerts its biological effects are not completely understood and there may be unknown beneficial activities.

Sodium nitroprusside (SNP) is a widely used NO donor chemical. NO generated from SNP has been shown to induce apoptosis in various

* Corresponding author at: School of Pharmacy Sungkyunkwan University Suwon city, Kyunggi-do, 440-746 Republic of Korea. Tel.: +82 31 290 7713; fax: +82 31 292 8800.
E-mail address: snpyo@skku.edu (S. Pyo).

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cultured cell types. In addition, administration of osteoblasts with SNP has been reported to affect osteoblastic metabolism [18]. The objective of this study was to investigate the ability of kob A to influence NO-mediated apoptosis and elucidate the cellular signaling and molecular mechanisms responsible for pharmacological activity by kob A in human osteoblast-like MG-63 cells.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise indicated, all the chemicals were purchased from Sigma Chemical Co. (St Louis, MO). Antibodies against Bcl-2, Bcl-X_L, Bax, p-c-Jun, estrogen receptor(ER)-α, ER-β, c-Jun, lamin A and NF-κB were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). JNK and p-JNK (Thr183/Tyr185) antibodies were purchased from Cell signaling Technology (Beverly, MA) CMH_2-DCFDA, Fluo-3 and Mito-Tracker Red CMXRos were obtained from Molecular Probes-Invitrogen (Eugene, Oregon). ICI182,780 was purchased from Tocris Cookson Ltd. (Bristol, UK).

2.2. Extraction and isolation of kob A

Kob A was extracted and purified from the dried roots of *Caragana sinica* as previously described [15]. The chemical structure of kob A is [3,4′-bibenzofuran]-6,6′-diol, 3′-(3,5-dihydroxyphenyl)-4-[(2S,3S,4R,5S)-4-(3,5-dihdroxyphenyl)tetrahydro-2,5-bis(4-hydroxyphenyl)-3-furanyl]-2,2′,3,3′-terahydro-2,2′-bis(4-hydroxyphenyl)-,(2S,2′R,3S,3′R) (Fig. 1). Kob A was identified through a comparison of its spectral data (NMR, IR, and UV) and $[\alpha]_D$ with the literature values [13]. The purity of the isolated compound was confirmed to be >99%. Kob A was dissolved in methanol to a concentration of 50 mg/ml, and was diluted to the appropriate concentrations with the culture medium.

2.3. Cell culture

Human osteoblast-like MG-63 cells (ATCC, Rockville, MD) were maintained in Dulbecco's modified Eagles medium supplemented with 10% heat-inactivated fetal bovine, L-glutamine (2 mM), penicillin (50 IU/ml), and streptomycin (50 μg/ml) (Gibco-BRL, Grand Island, NY). The cultures were maintained in a humidified atmosphere containing 5% CO2 at 37 °C. Medium was not supplemented with ascorbic acid and β-glycerophosphate, because the addition of these factors for the time period indicated did not change the characteristics of untreated or treated MG-63 cells.

2.4. Measurement of cell viability

MG-63 cells were seeded at a concentration of 2×10^4 cells/well in 96-well tissue culture plates and treated with various concentrations of SNP for the indicated times. In addition, to examine the protective effect of kob A, the cells were treated with 1 mM SNP in the absence or presence of kob A (25 and 50 μg/ml) for various times. Cell viability was measured by quantitative colorimetric assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], showing the mitochondrial activity of living cells. The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density at 550 nm using a Molecular Device microplate reader (Menlo Park, CA). The cell viability was expressed as the percentage of the untreated control.

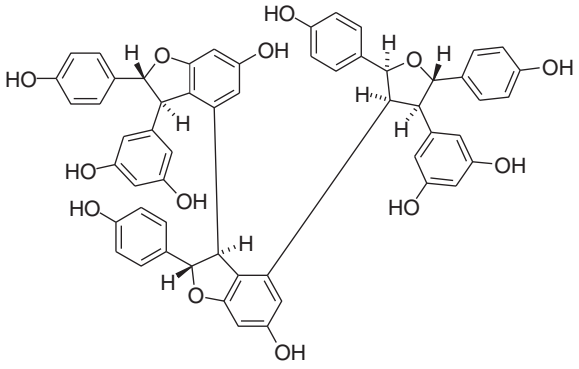

Fig. 1. Chemical structure of kobophenol A ($C_{56}H_{44}O_{13}$).

2.5. Assessment of nitric oxide (NO) production

The amount of NO_2^- accumulated in the culture supernatants was measured using the assay system reported by Ding et al. [19]. Briefly, 100 μl supernatant was removed from each well into an empty 96-well plate. After the addition of 100 μl Griess reagent to each well, absorbance at 540 nm was measured using a Molecular device microplate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve. The levels of absorbance are indicative of NO production. Griess reagent was prepared by mixing one part 0.1% naphthylethylenediamine dihydrochloride in distilled water plus one part 1% sulfanilamide in 5% concentrated H_3PO_4. The levels of nitric oxide were normalized by logarithmic transformation for multivariate analyses. Analyses were performed using the JMP™ software package (Version 4.01)(SAS Institute, Cary NC).

2.6. Morphological assessment of apoptosis

Cells undergoing apoptosis show characteristic morphologic changes such as shrinkage, chromatin condensation and DNA fragmentation. After MG-63 cells were incubated in the presence of 1 mM SNP with or without kob A (25 and 50 μg/ml) for 24 h, the cells were stained with Hoechst 33342 (10 μg/ml) and morphological changes were examined by fluorescence microscope. Cells with condensed chromatin or fragmented nuclei were considered to be apoptotic.

2.7. Determination of cell death by PI uptake assay and cell cycle analysis

Cell death was assessed by the PI uptake rate and the percentage of the sub-G1 populations through cell cycle analysis. The MG-63 cells were exposed to 1 mM SNP with or without kob A (25 and 50 μg/ml) for 24 h. The PI uptake was measured by collecting the cells and staining them with PI (1 μg/ml) for 15 min. The cells were then analyzed by flow cytometry using Win BRYTE HS (Bio-RAD, Richmond, CA). The Sub G_1 DNA content was determined using the following procedure. The cells were collected after treatment, washed twice with cold PBS and treated with 1 ml of cold citrated buffer (0.24 M sucrose, 40 mM sodium Citrate, pH 7.6) for 15 min. The cells were then centrifuged and suspended in 0.1 ml of a citrate buffer. Subsequently, 0.4 ml of a PI staining/lysis solution (0.5% NP-40, 0.5 mM EDTA) and 5 μl of a RNase A (10 mg/ml in TE, pH8.0) solution were added. The cells were incubated in the dark at room temperature for 30 min, and the position of the cell in the cell cycle was analyzed by flow cytometry. A minimum of 20,000 cells per sample was collected and the DNA histograms were further analyzed using ModiFitLT software (Verily Software House, Topsham, ME, USA).

2.8. Measurement of annexin V binding by flow cytometry

Annexin V/propidium iodide (PI) binding assay was employed to determine viable, early apoptotic cells. The MG-63 cells were treated with 1 mM SNP with or without Kob A (50 μg/ml) for 24 h. The cells were washed three times with PBS and stained with 5 μl Annexin V-FITC and 10 μl PI using ANNEXIN V-FITC APOPTOSIS DETECTION KIT I (BD-Pharmingen, San Diego, CA) according to the manufacturer's instruction.

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Subsequently, the fluorescence intensities of the PI and annexin V-FITC in 10,000 cells were analyzed by flow cytometry.

2.9. Measurement of the mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was determined using MitoTracker red (Molecular Probes, Eugene, Oregon). The MG-63 cells (5×10^5 cells) were seeded overnight in 6-well tissue culture plates and treated with 1 mM SNP with or without kob A (25 and 50 μg/ml) for 24 h. The MitoTracker Red CMXRos was added to cells at 100 nM final concentration. The cells were incubated for 15 min at 37 °C, washed with PBS, and fixed in 3% paraformaldehyde. After washing and centrifuging, the cell pellets were suspended with D-PBS buffer. The changes in MMP, as an intracellular red fluorescent intensity, were analyzed by flow cytometry.

2.10. Measurement of reactive oxygen species

Endogenous amounts of reactive oxygen species (ROS) were measured by a fluorometric assay with CMH_2-DCFDA; ROS sensitive dye was used to evaluate the intracellular ROS level. The MG-63 cells were treated with 1 mM SNP with or without kob A (50 μg/ml) for 8 h to determine the ROS level before severe cell death. The cells were then stained with 5 μM CMH_2-DCFDA for 15 min. After collecting the cells, the intracellular ROS level was measured by flow cytometry. At least 10,000 cells were analyzed. The level of intracellular ROS is expressed as a percentage of the untreated control.

2.11. Western blot analysis

MG-63 cells were incubated with 10 ml of a medium containing 1 mM SNP in the presence or absence of kob A (25 and 50 μg/ml) at 37 °C for various times in 100 mm culture dishes. The cells were collected by cell scraping and centrifuged at 500 g at 4 °C for 5 min. The cell pellet was washed twice with D-PBS, and the cells were resuspended in a homogenization buffer containing 50 mM Tris–Cl (pH 6.8), 2% SDS, and 1 mM DTT with the Halt protease inhibitor cocktail (PIERCE, Rockford, IL). Nuclear extracts were also prepared as previously described [20]. A Bio-Rad DC Protein assay kit (Bio-Rad Lab, Hercules, CA) was used to measure the protein concentration with BSA as the standard. The whole lysates (20 μg) and nuclear extracts (40 μg) were resolved on a 7.5% and 12% SDS-polyacrylamide gel, respectively. The fractionated proteins were electrophoretically transferred to an Immobilon polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL) and probed with the appropriate antibodies. The blots were then developed using an ECL kit (Amersham) according to manufacturer's instructions. In all immunoblotting experiments, the blots were reprobed with the anti-β-actin or anti-lamin A antibody (Sigma, St Louis, MO), which was used as a control for the protein loading.

2.12. Statistical analysis

Each result is reported as means ± S.E.M. All experiments were repeated at least three times independently. Two-way analysis of variance was used for analysis of differences among groups and the significant values are represented by an asterisk. ($^*p<0.05$).

3. Results

3.1. Kob A attenuated NO-induced cytotoxicity

MG-63 cells were exposed to various SNP concentrations, and the cell viability was assessed using a MTT assay. SNP treatment resulted in MG-63 cell death in a concentration- and time-dependent manner with increase of NO production (Fig. 2A and B). At concentrations higher than 1 mM, an apparent cell death (of approximately 83%) was detected within 24 h of incubation. When exposed to 1 mM SNP, the cells began to change their morphology at 12 h and the cell viability decreased with time. Thus, in all subsequent experiments, SNP was used at a concentration of 1 mM. The time of incubation and concentration of kob A used in these experiments had little effect on the cell viability (data not shown). Alkaline phosphatase (ALP) activity as marker for differentiation into functional osteoblasts was increased 11–35% in different concentration (5–50 μg/ml) of kob A than that of the control (0 μg/ml). The concentrations of kob A were selected based on previous reports (Lee et al., 2007). After confirming the lack of cytotoxicity, we next examined the capacity of kob A to protect cells from SNP-produced NO. As shown in Fig. 3A, coincubation of kob A with SNP significantly reduced cell cytotoxicity caused by SNP. A PI uptake assay was also carried out to examine the protective effect of kob A in SNP-induced cell death (Fig. 3B). The percentage of PI uptake in the SNP-treated MG-63 cells was 63.8% at 24 h. However, SNP treatment in the presence of kob A decreased the percentage of PI uptake (kob A, 25 μg/ml; 24.4% and kob A 50 μg/ml; 12.3%). These results correlated well with earlier findings obtained with MTT assay. To confirm the protective effects of kob A against NO-mediated cytotoxicity, we tested its effect on the cell death induced by different, structurally unrelated NO donors (SNAP and SIN-1). Kob A efficiently reduced the toxicity of NO donors, thus confirming the ability of kob A to partially inhibit the cytotoxic action of NO (Fig. 4).

To further determine if kob A can scavenge NO, the MG-63 cells were treated with 1 mM SNP in the absence or presence of kob A (25 and 50 μg/ml) for 24 h. Kob A did not induce significant NO production and did not have the capacity to scavenge NO in MG-63 cells. Therefore, the protective effect of kob A on the cell survival from SNP treatment might not be related to the NO scavenging effect of kob A, because kob A did not inhibit the production of NO and there was no apparent decrease in the NO level produced by SNP (Fig. 5).

3.2. Inhibition of NO-induced apoptosis by kob A treatment

The next set of experiments was performed to investigate whether kob A protects against SNP-induced apoptosis in MG-63 cells. In the cells exposed to a combination of two agents, we monitored apoptosis by analyzing the sub-G1 events and Annexin V-FITC binding in a cytofluorimeter and by nuclear staining with Hoechst 33342. When MG-63 cells were exposed to SNP for 24 h, a severe transition to the sub-G_1 cell cycle was observed and morphological alterations of the nuclei characteristic such as nuclear condensation for apoptosis were visible (Fig. 6A and B). In contrast, the increase in the percentage of cells in Sub G_1 of the cell cycle (60.2%) was decreased remarkably by the kob A treatment (kob A 25 μg/ml: 18.1%; kob A 50 μg/ml; 13.8%). Kob A also inhibited the formation of pre-apoptotic morphological changes in SNP-treated cells. Moreover, annexin V/PI binding assay showed that SNP increased the percentage of annexin V positive cells (68%), whereas only 18% of the cells treated with kob A (50 μg/ml) were annexin V positive (Fig. 6C). Taken together, these results indicate that SNP induces cell death through apoptosis and this SNP-induced apoptosis is significantly inhibited by kob A.

3.3. Effect of anti-estrogen ICI182.780 on the protective effect of kob A

Since kob A, one of the predominant oligostilbenes in the ethyl acetate extract of *C. sinica*, has estrogenic activity and is structurally similar to the estrogen 17β-estradiol (E2), it is possible that the protective effect of kob A may be mediated through estrogen receptors (ER). In order to examine this hypothesis, cells were pretreated with a pure estrogen antagonist, ICI182.780, for 2 h followed by cotreatment with SNP and kob A (Fig. 7A). The treatment of ICI182.780 did not abolish the protective effect of kob A. This antagonist alone did not cause reduced cell proliferation or increased cell death. In addition, the

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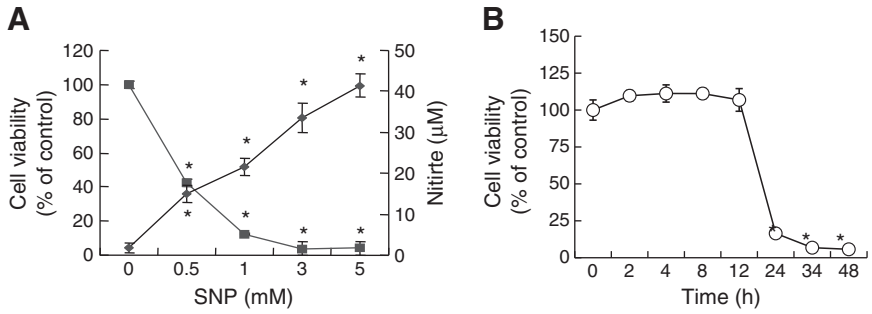

Fig. 2. Effects of SNP on cell viability and nitrite production. (A) MG-63 cells in 96 well culture plates (2×10^4 cells/well) were treated with various concentrations of SNP for 24 h. Cell viability (■) was measured by a MTT assay and nitrite levels (●) in the culture media were measured by Griess reaction. (B) Cells were treated with 1 mM SNP for indicated time points. Cell viability was measured after SNP treatment using MTT assay. Cell viability is presented as the percentage of untreated control cells. Data shown are the mean ± SEM of at least three independent experiments. *$P<0.05$ versus untreated control.

treatment of kob A did not affect the level of ER expression (Fig. 7B). These data suggest that the protective effect of kob A is not occurring in an ER-dependent manner.

3.4. Effects of kob A on SNP-induced apoptotic biochemical changes

Many of the biochemical changes are associated with apoptosis, such as changes in the production of reactive oxygen species (ROS) and the mitochondria membrane potential (MMP). To better understand how kob A protects cells against SNP cytotoxicity, we examined its effects on biochemical events during SNP-induced apoptosis. The change in the MMP level after SNP treatment with or without kob A was quantified by measuring the MMP level by flow cytometry after MitoTracker Red CMXRos staining. The SNP-exposed cells showed a significant decrease in the MMP level, but this decrease was inhibited by kob A (Fig. 8A and B).

In order to determine the effect of kob A on the intracellular ROS level, the MG-63 cells were exposed to SNP with or without kob A and the intracellular ROS level was measured by flow cytometry after staining with CMH_2-DCFDA. Exposure of MG-63 cells to SNP led to a significant increase in intracellular ROS level (Fig. 8C). However, treatment with kob A significantly decreased or quenched ROS formation. These results indicate that kob A acts as an ROS scavenger in this system.

3.5. Kob A inhibits JNK and p53 phosphorylation and NF-κB and c-Jun translocation by SNP

It has been suggested that JNK but not p38 MAPK or ERK is mainly involved in SNP-induced apoptosis in muscle cells and osteoblasts [21,22]. To evaluate the molecular mechanism of kob A's protection against SNP-induced apoptosis, we examined the response of the JNK pathway in co-treated cells with combination of SNP and kob A. After treating the MG-63 cells with SNP in the absence or presence of kob A (50 mg/ml), the level of JNK phosphorylation was assessed by Western blot analysis at the indicated times. As shown in Fig. 9A, SNP led to an increase in the levels of phospho-JNK and phospho-c-Jun in the cells, while treatment of cells with kob A markedly attenuated their phosphorylation level. Since it has been known that NF-κB and AP-1 also play an important role in NO-induced apoptotic cell death [23], we further determined whether kob A could inhibit NO-induced NF-κB and c-Jun levels in nuclei. As shown in Fig. 9B, kob A prevented the decrease of nuclear levels of NF-κB and c-Jun, AP-1 family. These results suggest that kob A prevents NO-induced JNK signaling and translocation of NF-κB and c-Jun.

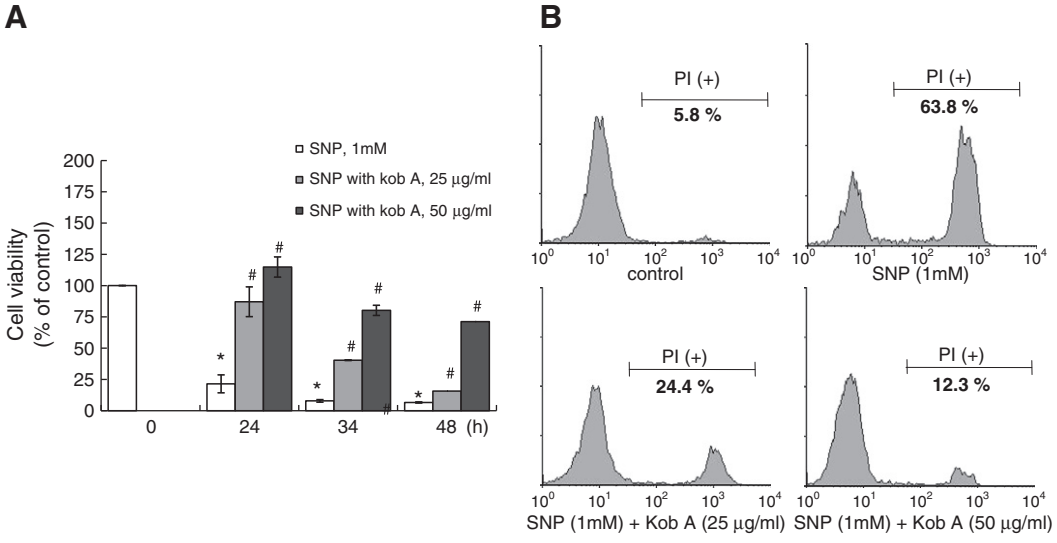

Fig. 3. Protective effects of kob A against SNP-induced cell death. (A) MG-63 cells were exposed to 1 mM SNP with or without different concentrations of kob A (25 and 50 μg/ml) for the indicated time. Cell viability is presented as the percentage of untreated control cells. Data shown are the mean ± SEM of at least three independent experiments. *$P<0.05$ versus untreated control; #$P<0.05$ versus 1 mM SNP-treated cells. (B) MG-63 cells were exposed to SNP (1 mM) in the presence or absence of Kob A for 24 h. After administration, the protective effects of Kob A against the SNP-induced MG-63 cell death were measured by the PI uptake. Data shown is representative of three independent experiments.

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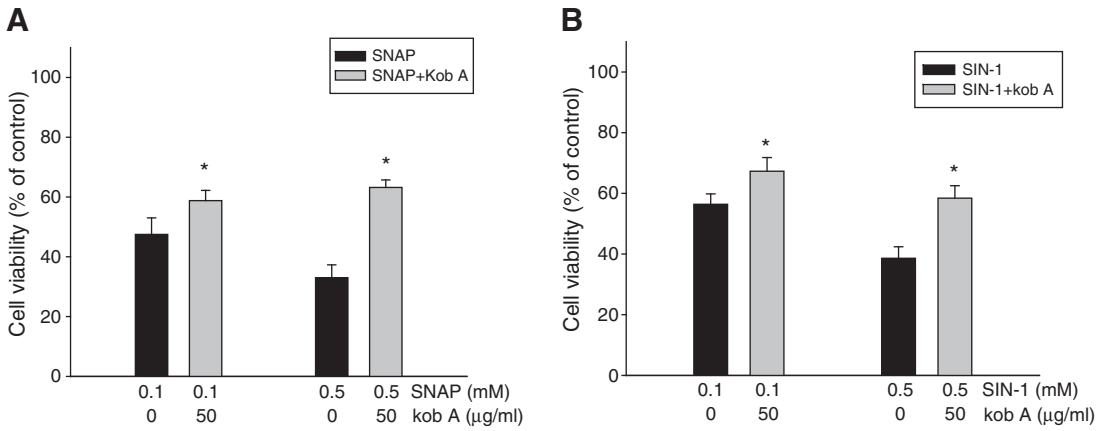

Fig. 4. Effects of kob A on NO generating compounds-induced cell death. MG-63 cells in 96 well culture plates (2×10^4 cells/well) were treated with different concentrations of SNAP (A) or SIN-1 (B) in the presence or absence of kob A (50 μg/ml) for 24 h. Cell viability was measured by a MTT assay. Cell viability is presented as the percentage of untreated control cells. Data shown are the mean ± SEM of at least three independent experiments. *$P<0.05$ versus SNAP-treated cells or SIN-1-treated cells.

3.6. Effects of kob A on apoptosis-associated gene expression

The anti-apoptotic effect of kob A when given to MG-63 cultures was further investigated by measuring the expression of genes involved in the execution of the apoptotic program. Apoptosis-associated gene profiles were determined for MG-63 cells incubated with SNP in the presence or absence of kob A. As shown in Fig. 9C, treatment with kob A prevented SNP-induced down-regulation of the anti-apoptotic Bcl-2 and Bcl-X_L levels. The expression of proapoptosis protein Bax increased significantly in SNP-treated group compared with that in the control group. However, kob A significantly decreased the level of Bax expression at 50 μg/ml.

4. Discussion

High NO concentrations from stimulation with pro-inflammatory cytokines, SNP or mechanical shear stress have potent inhibitory effects on osteoblast growth and differentiation [8], and can damage the osteoblasts. Postmenopausal bone loss is the result of an increase in bone turnover, which is estrogen dependent. Thus, excessive NO production can inhibit the proliferation of osteoblasts and accelerate osteoblast death resulting in the loss of bone mass. In this aspect, biological molecules that can protect the NO-induced osteoblast damage or cell death might be helpful in treating osteoblast death related disease.

The present study elucidates the ability and molecular mechanisms of kob A in inhibition of SNP-induced apoptosis in osteoblast-like MG-63 cells. Our results showing that SNP decreased cell survival and induced apoptosis of MG-63 cells are in keeping with the previous notion that treatment with SNP-induced rat osteoblast cell death [23,24]. However, cells exposed to kob A had significantly reduced proportion of apoptotic cells. Our preliminary experiments also demonstrated that kob A exhibited protective activity against serum withdrawal and necrotic stimuli from sodium azide and induced ALP activity which may lead to the prevention of cell death in MG-63 cells in this study (data not shown). In addition, the kob A treatment itself did not have any harmful effects in respect to cell growth and morphology. This in vitro evidence suggests that kob A might exert at least part of anti-apoptotic influence on the bone cells.

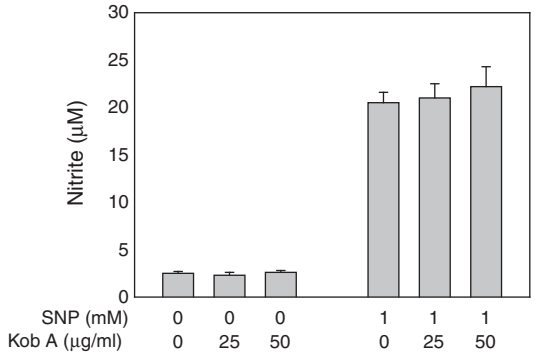

Fig. 5. Effects of SNP or/and kob A on nitrite production. MG-63 cells in 96 well culture plates (2×10^4 cells/well) were exposed to 1 mM SNP with or without different concentrations of kob A (25 and 50 μg/ml) for 24 h. Nitrite levels in the culture media were measured by Griess reaction. Data shown are the mean ± SEM of three independent experiments.

Various cell protective effects of estrogen, which are dependent on ER, are involved in neuroprotection, vascular protection and vasoprotective activity [6,25,26]. Since phytoestrogens such as kob A are structurally similar to the estrogen 17β-estradiol, we examined whether protective effect of kob A is dependent on estrogen receptor. The present data demonstrate that the protective effect of kob A was not reversed by the pure antagonist of estrogen ICI182.780, suggesting that the protective effect of kob A is not mediated through the estrogen receptor, and that additional mechanism of action through an alternative pathway is occurring.

The exogenous and endogenous forms of NO have similar effects in inducing a mitochondrial dysfunction and cell death in osteoblasts through an apoptotic pathway [24]. It is well known that the levels of intracellular ROS are augmented after administering SNP and ROS generation is intimately associated with certain functions in the early stages of apoptosis and mitochondrial dysfunction [12,27]. Our data also show that SNP treatment results in increase in ROS production. However, kob A blocked SNP-induced intracellular ROS level. Interestingly, our data showed that kob A was not effective in suppressing the SNP-induced production of NO. Hence, it is plausible that the inhibition of SNP-induced cell death by kob A is due to its suppressive activity at the ROS production stage rather than at the NO production stage. Furthermore, the inhibition of ROS production by kob A appears to be a specific effect, and the scavenging ability might represent one of the mechanisms by which kob A is cytoprotective under nitrosative stress. However, our results do not eliminate the possible involvement of alternative mechanisms, because ceramide is known to be involved in SNP-induced osteoblast apoptosis [28]. Therefore, the present data suggest that protective effect of kob A on SNP-induced cell death is at least partially mediated through suppression of ROS production.

Many independent lines of evidences suggest that ROS is a prerequisite for the collapse of MMP [29]. In the present study, our data demonstrate that the MMP collapsed as a result of nitrosative stress. However, kob A prevented this collapse of the MMP, suggesting

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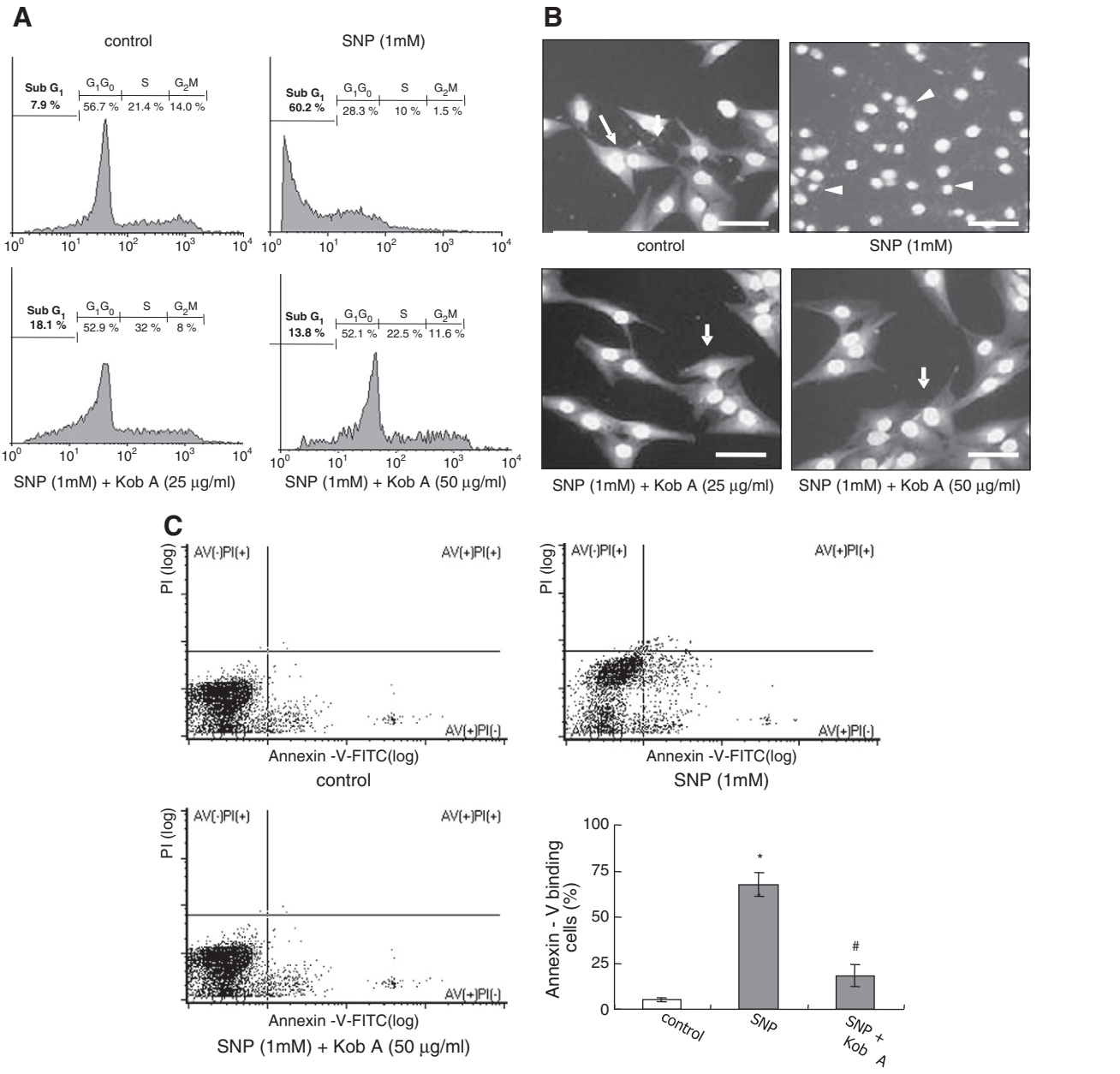

Fig. 6. Kob A inhibits SNP-induced MG-63 cell apoptosis assessed by apoptotic nuclei staining and flow cytometric analysis. (A) Cell cycle analysis after treatment with 1 mM SNP in the presence or absence of kob A for 24 h. The value in each treatment group represents the percentage of the sub-G1 fraction. (B) Hoechst 33342 staining after treatment with SNP in the presence or absence of kob A. The arrow indicates normal cells and arrowheads indicate the typical apoptotic nuclei (magnification, ×200). Scale bars equal 20 μm. (C) Distribution of viable, apoptotic and necrotic cells in the presence or absence of kob A as measured by annexin V/PI staining. The results are from one representative experiment of three. *$P<0.05$ versus control (no treatment); #$P<0.05$ versus 1 mM SNP-treated cells.

that kob A inhibits the mitochondrial collapse and apoptosis by scavenging the intracellular ROS and that the protective effect of kob A is related to its antioxidant property. It is also known that the level of ROS production is increased by oxidative stress, which influences the lysosomal degradative mechanisms [30]. Thus, it is possible that internal generation of ROS by SNP might be inhibited by kob A through lysosomal stabilization. Indeed, the results of this study have shown that the intracellular ROS produced by SNP damaged the integrity of the lysosomal membrane (data not shown), which induces lysosomal rupture. This process can be prevented by kob A treatment, suggesting that kob A treatment sustains the integrity of the lysosomal membrane.

It has been known that generation of ROS, together with the release of cytochrome c and pro-apoptotic proteins from mitochondria, triggers caspase activation and apoptosis [31]. In this study, the activity of casapse-3 was measured in order to determine if the kob A treatment blocks the activation of caspase-3. We observed that there was no significant change in caspase-3 activity after the SNP treatment with or without kob A (data not shown). This suggests that the activation of caspase-3 is not involved in this experiment model, or the high toxicity of SNP at given dose can kill the cells before initiating the caspase-cascade. In addition, it is possible that kob A prevents a distinct, caspase-independent pathway of SNP-induced cell death.

The MAP kinase family, including p38 MAP kinase, JNK, and ERK, has fundamental roles in both maintaining cell survival and the induction of cell death [32]. Among the MAP kinases, the JNK cascade is most sensitive to ROS, which induce the long-term activation of JNK. c-Jun and ATF-2, which are transcription factors, are the main substrates for JNK protein kinase (Turapev, 2002). Recent data suggests that JNK but not p38 MAPK or ERK is mainly involved in

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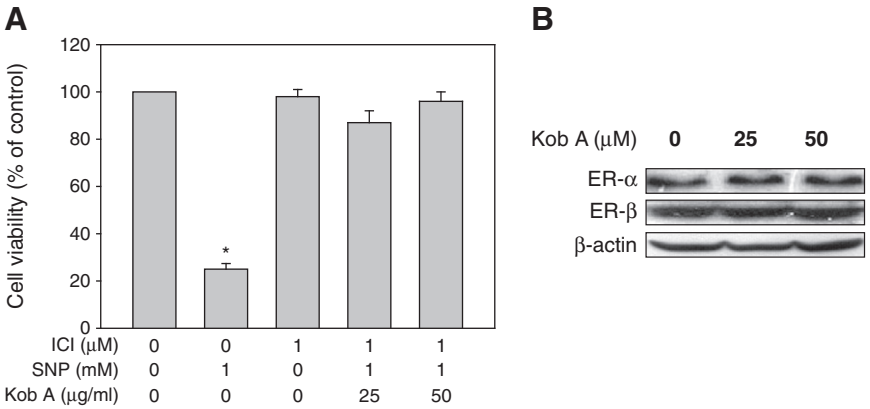

Fig. 7. Lack of effect of anti-estrogen ICI182.780 on the protective effect of kob A. (A) MG-63 cells were pretreated with or without ICI182.780 (1 μM) for 2 h, followed by treatment with SNP (1 mM) in the presence or absence of kob A (25 and 50 μg/ml) for 24 h. Cell viability is presented as the percentage of untreated control cells. Data shown are the mean ± SEM of at least three independent experiments. *$P<0.05$ versus untreated control. (B) Estrogen receptor expression in MG-63 cells. Estrogen receptor level was detected from whole cell lysates with Western blotting. Equal amount of protein was used in each sample and β-actin was used as a loading control. Similar results were obtained in two additional experiments.

SNP-induced apoptosis in muscle cells and osteoblasts [21,22]. Therefore, this study examined the potential involvement of the JNK signaling pathway in kob A-mediated cytoprotection. The present results demonstrate that SNP activated the JNK signaling pathway, which can induce the phosphorylation of c-Jun leading to cell apoptosis. However, kob A inhibited the phosphorylation of JNK and c-Jun, implicating that kob A-mediated osteoprotection is linked to the inhibition of JNK signaling pathway. Moreover, we demonstrate that treatment with kob A resulted in inhibition of SNP-induced nuclear levels of NF-κB and c-Jun, a member of AP-1. Based on these findings, our results suggest that inhibition of SNP-mediated reduction of the JNK, NF-κB and AP-1 activities by kob A treatment leads to a protective effect on the NO-induced osteoblast damage.

SNP regulates the expression of Bcl-2 protein family in osteoblast cells [24]. In this study, the involvement of the Bcl-2 protein family in NO-induced apoptosis was demonstrated by examining the effect of SNP on the expression of apoptosis-related genes. We also showed that kob A can elevate the expression of Bcl-2 and Bcl-X_L proteins that

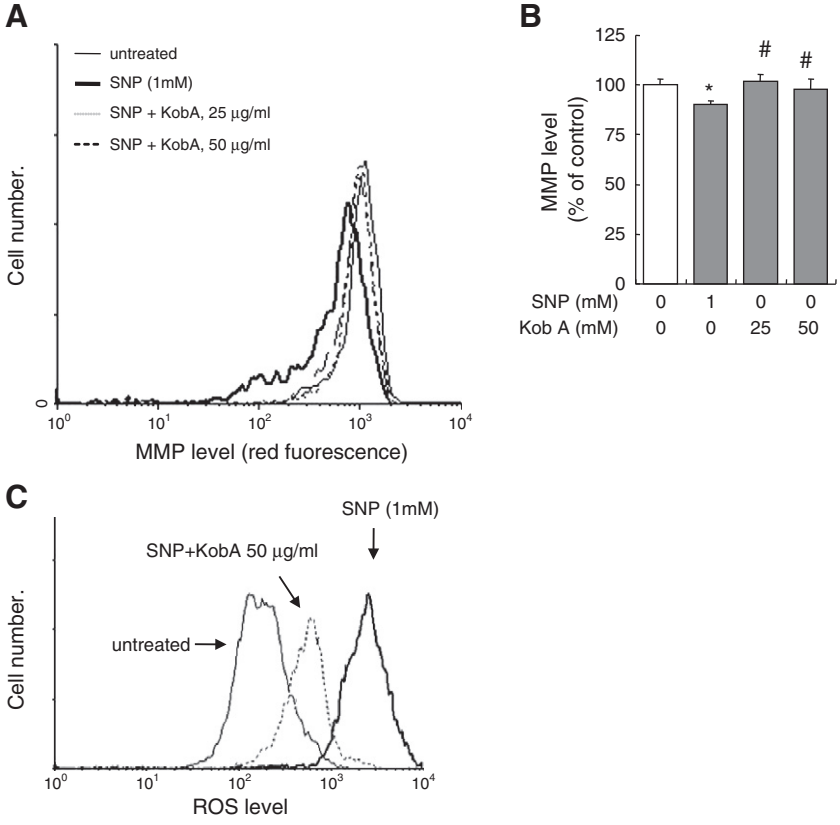

Fig. 8. Effect of kob A on decrease of MMP and increase of ROS in SNP-treated cells. MG-63 cells were exposed to 1 mM SNP with or without indicated concentrations of kob A for 24 h. (A) Mitochondrial membrane potential was measured using a fluorescent cationic probe rhodamine-123 by flow cytometer with FL-1 filter after MTR-CMXRos staining and (B) the results are expressed as a percentage of the untreated control. Data shown are the mean ± SEM of at least three independent experiments. *$P<0.05$ versus untreated control; #$P<0.05$ versus 1 mM SNP-treated cells. (C) The intracellular ROS production was detected by CMH_2-DCFDA method. The results are from one representative experiment of three. Data shown is representative of three independent experiments.

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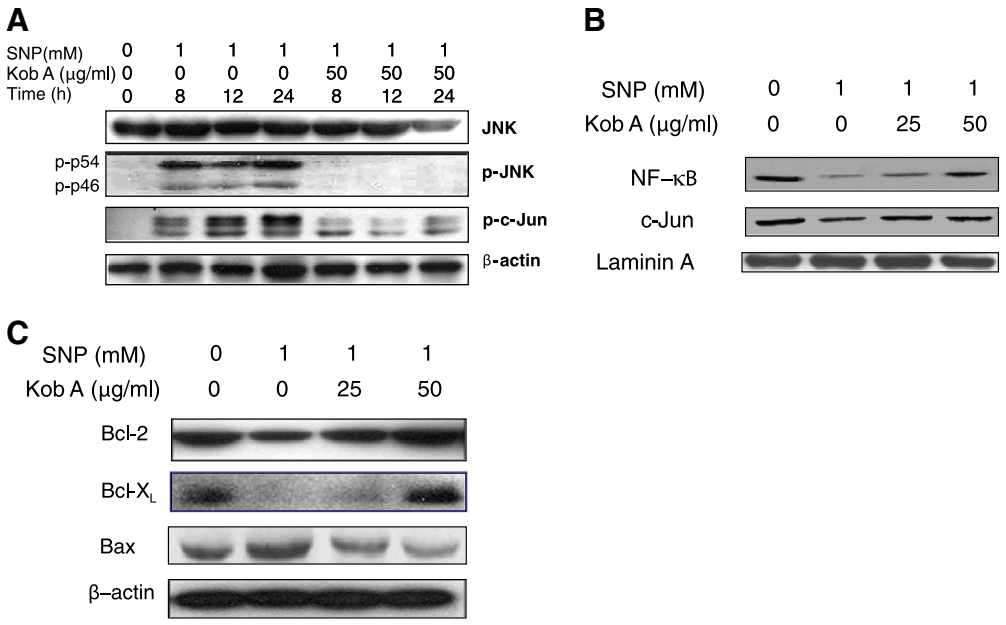

Fig. 9. Immunoblot analysis on Bcl-2 family, phosphorylation of JNK, NF-κB and c-Jun in response to SNP and kob A. (A) MG-63 cells were treated with 1 mM SNP in the presence or absence of Kob A (50 μg/ml) for indicated times. Levels of phosphorylated JNK and c-Jun were immunodetected. (B) MG-63 cells were treated with 1 mM SNP in the presence or absence of Kob A (50 μg/ml) for 4 h. (C) MG-63 cells were exposed to 1 mM SNP with or without Kob A (50 μg/ml) for 24 h. Bcl-2, Bcl-X_L and Bax protein levels were measured by Western blotting. Levels of nuclear NF-κB and AP-1 were immunodetected. Amounts of β-actin and lamin A were determined as the internal standard. Data shown is representative of three independent experiments.

are decreased by SNP, while the level of Bax expression is decreased by kob A treatment. These results suggest that kob A inhibits osteoblast cell death by increasing the production of the anti-apoptotic proteins, thereby preventing apoptosis.

It has been known that stilbenoids from Caragana species have demonstrated anticancer activity in vitro and in vivo [33]. Some of oligostilbenes also showed strong anti-inflammatory effects in vivo. Although the pharmacological in vivo studies of kob A have been limited, our data suggest, in conjunction with previous findings, that a modest kob A intake may significantly suppress NO-induced host cell death.

In conclusion, kob A can inhibit the NO-induced apoptosis of osteoblasts. This protective effect may not be related to its ability to scavenge NO, but may be related to its ability to scavenge intracellular ROS, prevent the decrease in the MMP, down-regulate Bcl-2, and inhibit the activation of the JNK signaling pathway and reduction in NF-κB and AP-1 activities. Thus, results of this study show a novel biological function for kob A and suggest that kob A may function as an effective therapeutic agent for the management of osteoporosis.

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