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Agastache rugosa Leaf Extract Inhibits the iNOS Expression in ROS 17/2.8 Cells Activated with TNF-α and IL-1β

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It has been suggested that nitric oxide (NO) derived from inducible nitric oxide synthase (iNOS) may act as a mediator of cytokine-induced effects on bone turn-over. NO is also recognized as an important factor in bone remodeling, i.e., participating in osteoblast apoptosis in an arthritic joint. The components of *Agastache rugosa* are known to have many pharmacological activities. In the present study, we investigated the effects of *Agastache rugosa* leaf extract (ELAR) on NO production and the iNOS expression in ROS 17/2.8 cells activated by a mixture of inflammatory cytokines including TNF-α and IL-1β. A preincubation with ELAR significantly and concentration-dependently reduced the expression of iNOS protein in ROS 17/2.8 cells activated with the cytokine mixture. Consequently, the NO production was also significantly reduced by ELAR with an IC_{50} of 0.75 mg/mL. The inhibitory mechanism of iNOS induction by ELAR prevented the activation and translocation of NF-κB (p65) to the nucleus from the cytosol fraction. Furthermore, ELAR concentration-dependently reduced the cellular toxicity induced by sodium nitroprusside, an NO-donor. These results suggest that ELAR may be beneficial in NO-mediated inflammatory conditions such as osteoporosis.

Key words: Nitric oxide, Osteoarthritis, Bone turn-over, Osteoblast, *Agastache rugosa*

INTRODUCTION

Inflammatory conditions such as rheumatoid arthritis (RA), which are characterized by local osteolysis, are associated with the activation of inducible nitric oxide synthase (iNOS) (Grabowsk *et al*., 1996; Boileau *et al*., 2002). Proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α can act as powerful local stimulatory signals for bone resorption (Gowen *et al*., 1983; Bertrolini *et al*., 1986). These factors function via interaction with receptors on the osteoblasts, which modulate osteoclast function by the release of unknown signals named osteoclast resorption stimulating activity (McSheehy and Chambers, 1986). IL-1β and TNF-α may also inhibit osteoblast activity, suggesting a mechanism for uncoupling bone resorption and deposition during inflammation. Under physiological conditions, these processes are carefully coordinated such that deposition is coupled to resorption. In metabolic and inflammatory bone diseases, these processes may become uncoupled, resulting in loss of bone mass as can be seen in postmenopausal osteoporosis, Paget's disease of bone and RA. Activation of iNOS by inflammation also has been related with apoptotic cell death in many cells (Armour *et al*., 2001). Thus, nitric oxide (NO) produced by proinflammatory cytokines in osteoblasts is also one of the possible factors that cause apoptosis (Mogi *et al*., 1999, 2000). Transcriptional control of osteoblast apoptosis has been reported, in which nuclear factor-kappa B (NF-κB) and/or activating protein-1 (AP-1) seems to be an important apoptotic signal pathway (Kitajima *et al*., 1996; Chae *et al*., 2000a, 2000b). The rat osteoblast-like, osteosarcoma cell line, ROS 17/2.8, is a well-established cell line that has been extensively used experimentally.

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ROS 17/2.8 cells possess a proliferative capacity that characterizes immature osteoblasts; and they also express differentiated proteins that characterize mature osteoblast; for example, alkaline phosphatase (Rodan *et al*., 1991) and bone morphogenic protein-2 (Yamaguchi *et al*., 1991; Wang *et al*., 1990). Earlier studies indicate that components of *Agastache rugosa* have various pharmacological actions such as antifungal (Shine, 2004) and anti-HIV integrase actions (Kim *et al*., 1999). So far, the effect of *Agastache rugosa* leaf extract (ELAR) on the iNOS expression in osteoblasts has not been reported. In the present study, we investigated whether ELAR inhibits NO production in ROS 17/2.8 cells when activated with inflammatory cytokines including IFN-γ and TNF-α by suppressing the induction of the iNOS gene. We found that ELAR concentration-dependently reduced the iNOS expression by inhibiting NF-κB translocation, and it increased cell survival from NO-induced toxicity. Our results thus suggest that ELAR may be beneficial in the prevention of osteoporosis and inflammation related arthritis.

MATERIALS AND METHODS

Materials

Sulphanilamide, *N*-(1-naphthyl)ethyleneamine, leupeptin, pepstatin A, and phenylmethylsulfonylfluoride were from Sigma (St. Louis, MO, USA). IL-1β and TNF-α were from R&D Systems, Inc. (Minneapolis, MN, USA). An inducible NOS antibody was from Transduction Laboratories (Lexington, KY, USA). An NF-κB antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase labeled goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). An ECL Western blotting detection reagent was from Amersham Pharmacia (Buckinghamshire, UK). The extract of *Agastache rugosa* was obtained from standard extraction method published elsewhere (Kim *et al*., 1999).

Cell culture

ROS 17/2.8 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in Dulbecco's Modified Eagle medium (DMEM) supplemented with 25 mM *N*-(2-hydroxyethyl) piperazine-*N*-2-ethanesulphoneic acid (HEPES), 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum.

Cell stimulation

The cells were stimulated with cytokine mixture (CM) containing IL-1β (25 ng/mL) and TNF-α (50 ng/mL) for 24 or 48 h, and ELAR (0.5-2 mg/mL) was added simultaneously or 1 h prior to CM administration. ELAR was dissolved in sterile distilled water and filtered through a 0.2 μm filter.

Assay for nitrite production

NO was measured as its stable oxidative metabolite, nitrite, as described previously (Green *et al*., 1981). At the end of the incubation, 100 μL of the culture medium was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride, and 1% sulfanilamide in 5% phosphoric acid). The absorbance at 550 nm was measured, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

Assay for iNOS protein

iNOS protein was analyzed by immunoblotting with the anti-iNOS antibody as described previously (Kang *et al*., 1999). Briefly, cells were harvested in a buffer containing 50 mM Tris-Cl, 1 mM EDTA, 1 mM leupeptin, 1 mM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol at pH 7.5. The cells were then sonicated. The harvested cells were then centrifuged at 7,500 × g for 15 min, and the supernatants were subjected to SDS-PAGE (7.5% gel). The separated proteins were electrophoretically transferred to PVDF membranes, and the membranes were incubated with the anti-iNOS antibody for 2 h followed by peroxidase-labeled goat anti-rabbit IgG for 1 h. Antigen-antibody complexes were detected using ECL Western blotting detection reagents (Amersham) according to the manufacturers instruction.

Cell respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of MTT to formazan. Cells in 24-well plates were incubated (37°C) with MTT (0.2 mg/mL) for 60 min. Culture medium was removed by aspiration and cells were solubilized in DMSO. Changes in absorbance at a wavelength of 570 (OD_{570}) were measured by a microplate reader (Bio-Rad Model 550).

Electrophoretic mobility shift assay (EMSA)

Cells were plated at a density of 1×10^7 cells per 100 mm dish. The cells were rinsed with fresh medium and preincubated with ELAR for 1 h before addition IL-1β plus TNF-α. The cells were washed three times with cold phosphate-buffered saline (PBS) and harvested by scraping into 5 mL of PBS, and then pelleted at 2,000 rpm for 5 min. The pellet was resuspended in 1 mL of ice-cold lysis buffer (10 mM Tris-Cl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/mL

leupeptin, 5 μg/mL pepstatin, and 5 μg/mL aprotinin at pH 7.4) and incubated for 15 min on ice with occasional vortexing. After centrifugation at 3,500 rpm for 5 min, nuclei were washed with 1 mL of washing buffer (10 mM HEPES-KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 5 μg/mL leupeptin, 5 μg/mL pepstatin, and 5 μg/mL aprotinin at pH 7.9) and 30-50 μL of hypertonic buffer (20 mM HEPES-KOH, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 5 μg/mL leupeptin, 5 μg/mL pepstatin, and 5 μg/mL aprotinin at pH 7.9) was added to the nuclear pellet and incubated on ice for 40 min with constant shaking. Nuclear proteins were isolated by centrifugation at 14,000 rpm for 30 min. Protein concentrations were determined by Bradford assay with aliquots. Nuclear extracts (5 μg) were stored at -70 °C until used for electrophoretic mobility shift assay (EMSA). Five mg of nuclear protein and 1 mg of poly(dI-dC) per reaction were incubated for 15 min at room temperature with NF-κB consensus sequence (Santa Cruz Biotechnology, Santa Cruz, CA) that was 3'-end labeled with γ-^{32}P-dATP. The sequence of oligonucleotide NF-κB probe was 5'-CAAAACAGGGGCTTTCCC-TCCTCA-3'. After the binding reaction, samples were analyzed by electrophoresis on a 6% native polyacrylamide gel that was run in 0.5 × Tris borate-EDTA (TBE) buffer at pH 8.0. The dried gel was then subjected to autoradiography.

Western blot for translocation of NF-κB

Cells were plated in 60-mm diameter culture dishes at a density of 5×10^6 cells and allowed to adhere overnight. Thereafter, medium was added, and the cells were treated with various concentrations of ELAR for 1 h before the addition of cytokines. After incubation, the cells were collected into the buffer solution and sonicated. Proteins were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked for 1 h in TTBS containing 5% nonfat dry milk and incubated for 1.5 h in primary antisera (anti-rabbit NF-κB, 1:1000) containing 5% nonfat dry milk. The blots were washed four times with TTBS (5 min/wash) and incubated for 45 min at room temperature in horseradish peroxidase-conjugated anti-rabbit secondary antibody at a dilution of 1:5,000. The blots were washed again three times with TTBS at room temperature and the proteins (NF-κB) were detected by using ECL reagents.

Statistical evaluations

Data are expressed as the mean ± SEM of results obtained from the number (n) of experiment. Differences between data sets were assessed by one way analysis of variance (ANOVA) followed by Dunnett's test. A level of $P<0.05$ was accepted as statistically significant.

RESULTS

Effects of a combination of the cytokine mixture on NO production and the iNOS protein expression

In ROS 17/2.8 cells, the amount of NO produced by each cytokine was almost the same as that produced by the vehicle-treated control when cultured up to 48 h regardless of concentrations added. However, when IL-1β and TNF-α were combined altogether, NO production was increased in a time and concentration-dependent manner. For example, the amount of NO (measured as nitrite) was 2 ± 0.002 μM in control media, which was increased to 9.2 ± 0.006 μM by the combination of CM for a 48 h incubation. The expression of iNOS protein was also concentration-dependent (Fig. 1A and B). Fig. 1C shows the time-dependent expression of iNOS by CM treatment.

Effects of ELAR on the production of NO and the iNOS protein expression

Fig. 2A and B show the inhibitory effect of ELAR on the production of NO and the iNOS protein expression. As the concentration of ELAR increased, the iNOS expression was diminished, resulting in a decrease of NO production. ELAR inhibited NO production by 50% (IC_{50}) at a concentration of 0.75 ± 0.11 mg/mL. To verify whether the reduced NO production and diminished expression of iNOS at a higher concentration of ELAR (2 mg/mL) occurred by non-specific action such as the cellular toxicity of the compound, cell viability was measured. As shown in Fig. 2C,

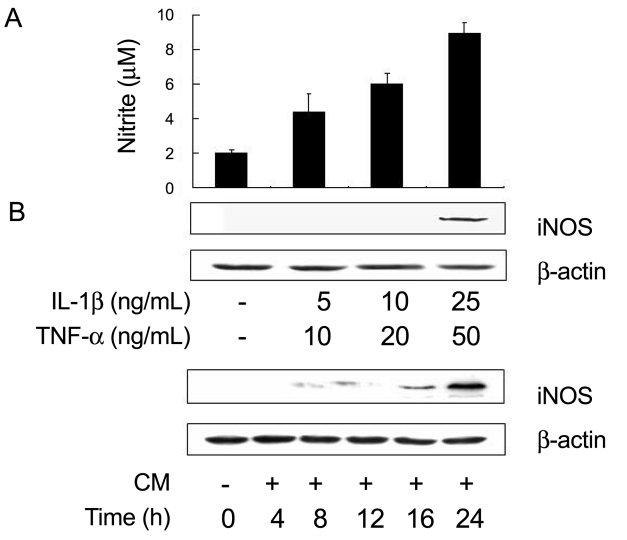

Fig. 1. Time- and concentration-dependent iNOS expression and NO production. (A) Nitrite quantitation. Cells were incubated for 48 h to determine the nitrite concentration with different combination of cytokines as indicated. (B) Western blot. Cells were incubated for 24 h and iNOS protein was analyzed by western blot. Time - dependent expression of iNOS protein by the cytokine mixture containing IL-1β (25 ng/mL) and TNF-α (50 ng/mL).

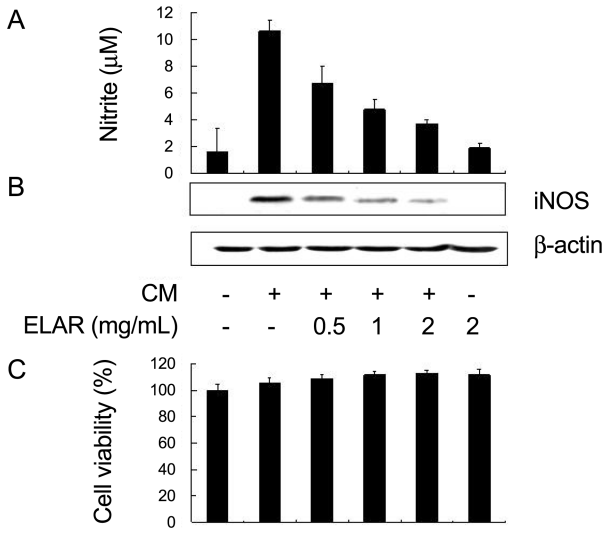

Fig. 2. Effects of ELAR on the iNOS expression and NO production in ROS 17/2.8 cells. Cells were preincubated with ELAR for 1 h and then treated with CM for 24 h or 48 h. (A) Nitrite quantitation. (B) Western blot with the iNOS antibody. (C) Cell viability determined by an MTT assay.

ELAR did not significantly change the cell viability up to 2 mg/mL for 24 h. Therefore, the reduced NO production as well as the decreased iNOS protein expression by ELAR was not due to a toxic action of ELAR on the cells.

Inhibition of NF-κB by ELAR in ROS 17/2.8 cells activated by CM

To determine the effect of ELAR on NF-κB activity, we determined the NF-κB activity in nuclear extract of cells challenged with CM for 1 h. As shown in Fig. 3, CM caused a significant increase in the level of the NF-κB-DNA complex, which was decreased by treatment with ELAR in a concentration-dependent manner. As shown in Fig. 4, ELAR also inhibited translocation of NF-κB (p65) from the cytosol to the nucleus in a concentration-dependent manner.

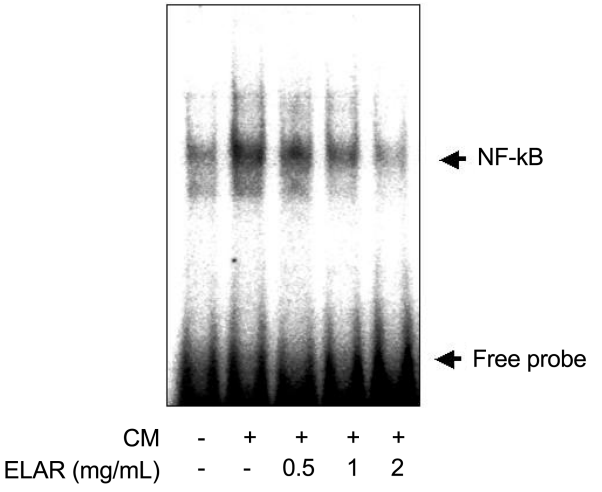

Fig. 3. Inhibition of NF-κB activity by ELAR in ROS 17/2.8 cells activated with CM. Cells were stimulated with CM for 1 h in the absence or presence of ELAR. Nuclear extracts from these cells were obtained and combined with a labeled NF-κB oligonucleotide probe.

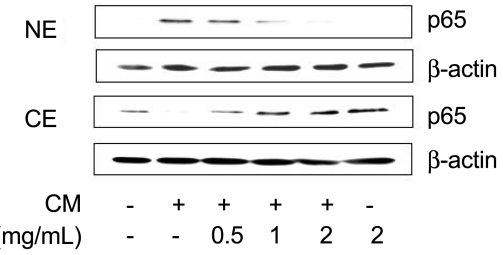

Fig. 4. Inhibition of p65 translocation by ELAR in ROS17/2.8 cells. Cells were stimulated with CM for 1 h in the presence of ELAR (0.52 mg/mL). Cytosolic extract (CE) and nuclear extract (NE) were isolated and investigated as to whether ELAR inhibited NF-κB translocation.

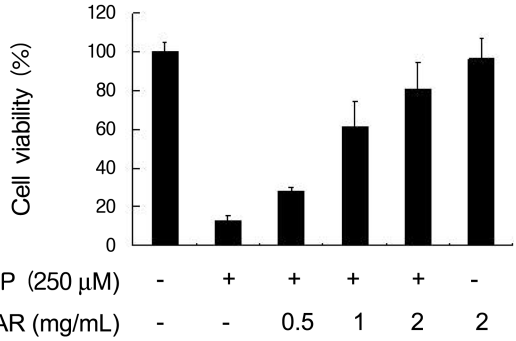

Fig. 5. Protective effects of ELAR against NO-mediated cell damage. Cells were pre-incubated with different concentrations of ELAR for 1 h in 5% serum media and then treated with SNP (250 μM) for 12 h. After completion of incubation, cells were subjected to an MTT test for cell viability.

Protective effects of ELAR against NO-mediated cellular damages

As shown in Fig. 5, ELAR increased the cell viability in ROS cells in the presence of SNP, a well-known NO-donor. Cells treated with 250 μM SNP for 12 h showed a 13 ± 2% survival rate. However, in the presence of a different concentration of ELAR, a reduced survival rate due to SNP was recovered in a concentration-dependent manner. At 2 mg/mL of ELAR, the survival rate was increased up to 82 ± 12%. However ELAR alone did not damage cell viability.

DISCUSSION

In this study, we demonstrated that ELAR inhibits iNOS induction in ROS 17/2.8 osteoblast cells activated with pro-inflammatory cytokines. Cytokines can induce the ex-

pression of the iNOS isoform in many cell types, including osteoblasts (Armour *et al*., 1999). Although supporting data are not shown here, we found that single cytokines had little or no stimulatory effect on NO production in ROS 17/2.8 cells. However, the combination of IL-1β and TNF-α caused a dramatic stimulation of NO production. In human osteoblast (hOB)-like cells, a single cytokine also does not increase NO production (Macpherson *et al*., 1999); thus this effect seems to be common. In a murine iNOS gene, cytokine responsive elements for the binding of transcriptional factors such as AP-1 and NF-κB are present, and NF-κB activation is known to be required for cytokine-induced iNOS mRNA expression (Niederberger *et al*., 2003). NF-κB activation is also essential for the induction of iNOS in osteoblasts. The concentration-dependent reduction of NO production by ELAR can be explained as the result of NF-κB inactivation. In deed, it inhibited the translocation of activated NF-κB from the cytosol to the nucleus. On the other hand, NO has been associated with a marked inhibition of osteoblast cell proliferation (Hukkanen *et al*., 1995), pathogenesis of arthritis. High levels of nitrite/nitrate are present in the serum and synovial fluid of arthritis patients, and the mRNA and protein of iNOS have been detected in the synovial tissues of both osteoarthritis and RA patients (Sandhu *et al*., 2003). In the present study, ELAR significantly reduced the NO-induced cell death from the cytokine mixture, suggesting that it may prevent apoptotic cell death resulting from excess NO. Recently NO was reported to enhance bone loss by augmenting the cytokine-induced MMP-1 production in osteoblasts (Lin *et al*., 2003). Furthermore, NO acting as a paracrine factor is also recognized as an important factor in bone remodeling, i.e., in osteoblast apoptosis (Armour *et al*., 2001) and in cartilage loss and degradation in an arthritic joint as apoptosis of chondrocytes (Pelletier *et al*., 2001). Apoptosis in osteoblasts is usually not observed under normal bone remodeling. However, inflammatory conditions such as RA, which are characterized by local osteolysis, are associated with apoptotic cell death of osteoblast. Despite being a transformed cell line, the ROS 17/2.8 cell is able to undergo NO-induced apoptosis (Jilka *et al*., 1998) with modification of serum restriction. ROS 17/2.8 cells represent a highly differentiated stage of the osteoblastic lineage despite their transformed phenotype, expressing both osteoclacin (Ihbe *et al*., 1998) and the E11 antigen defining the osteoblast-osteocyte transition (Wetterwald *et al*., 1996), and can serve a good model for investigating the mechanisms of osteoblastic apoptosis (Ihbe *et al*., 1998). Because of evidence demonstrating apoptosis is the fate of the majority of osteoblasts (Jilka *et al*., 1998), it is highly plausible that prevention of apoptosis should render the cell to increase the number of functional osteoblasts which would prolong the bone formation period. A recent publication shows that agastinol and agastenol, components of ELAR, inhibit apoptosis in U937 leukemia cells by modulation of caspase-3 (Lee *et al*., 2002). This further supports our speculation; however, further study is required to determine whether ROS 17/2.8 cells induce apoptosis by a cytokine mixture or excess NO, in which ELAR prevents NO-mediated apoptosis. In conclusion, we investigated the ability of ELAR to inhibit the iNOS expression in osteoblast cells. We found that ELAR significantly reduced NO production by preventing NF-κB translocation. Thus, it may protect osteoblast cells from a highly toxic NO environment such as that found in an inflammation condition. Therefore, the present data demonstrate that ELAR may be beneficial against NO-mediated inflammatory bone disorders such as RA or osteoporosis.

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