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Inhibition of Inducible Nitric Oxide Synthase Expression by YS 49, a Synthetic Isoquinoline Alkaloid, in ROS 17/2.8 Cells Activated with TNF-α, IFN-γ and LPS

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Nitric oxide (NO) has been suggested to act as a mediator of cytokine-induced effects of turn over of bone. Activation of the inducible nitric oxide synthase (iNOS) by inflammation has been related with apoptotic cell death in osteoblast. YS 49, a synthetic isoquinoline alkaloid, inhibits NO production in macrophages activated with cytokines. In the present study, we investigated the molecular mechanism of YS 49 to inhibit iNOS expression in ROS 17/2.8 cells, which were activated with combined treatment of inflammatory cytokines (TNF-α, IFN-γ) and lipopolysaccharide (LPS). Results indicated that YS 49 concentration-dependently reduced iNOS mRNA and protein expression, as evidenced by Northern and Western blot analysis, respectively. The underlying mechanism by which YS 49 suppressed iNOS expression was not to affect iNOS mRNA stability but to inhibit activation and translocation of NF-κB by preventing the degradation of its inhibitory protein IκBα. As expected, YS 49 prevented NO-induced apoptotic cell death by sodium nitroprusside. Taken together, it is concluded that YS 49 inhibits iNOS expression by interfering with degradation of phosphorylated inhibitory κBα (p-IκBα). These actions may be beneficial for the treatment of inflammation of the joint, such as rheumatoid arthritis.

Key Words: Tetrahydroisoquinoline, Inducible nitric oxide synthase, Osteoblast, Nuclear kappa B, Cytokines

INTRODUCTION

The adult human skeleton is continually renewed by temporary anatomic structures, such as juxtaposed osteoclasts and osteoblasts; two specialized cell types that originate from progenitors residing in the bone marrow. Orderly supply of osteoclasts and osteoblasts is evidently essential for skeletal homeostasis, as changes in their number are largely responsible for the mismatch between bone formation and resorption that underlies most systemic or localized bone diseases, including osteoporosis (Manolagas et al, 1995; Jilka et al, 1992, 1998; Weinstein et al, 1998). Inflammatory diseases such as rheumatoid arthritis (RA) are associated with increased production of nitric oxide (NO), resulting from activation of the inducible nitric oxide synthase (iNOS) pathway (Farrell et al, 1992; Grabowski et al, 1996a; Hilliquin et al, 1997). Studies in animal models have suggested that NO plays a causal role in the pathogenesis of joint inflammation and tissue damage, since the severity of arthritis can be reduced by the administration of NOS inhibitors (Ialenti et al, 1993; McCartney-Francis et al, 1993; Stefanovic-Racic et al, 1994). Several types of cells present in the joint, including synovial fibroblasts, endothelial cells and chondrocytes, can be induced by pro-inflammatory cytokines to produce NO *in vitro* (Stadler et al, 1991; Palmer et al, 1993; Ralston et al, 1994; Grabowski et al, 1996b). Moreover, localization studies have shown that iNOS expression was up-regulated in synovial lining cells, chondrocytes and blood vessels in joint tissues obtained from patients with RA (Stefanovic-Racic et al, 1994; Sakurai et al, 1995; Grabowski et al, 1997). The localization of iNOS expression to the synovial lining layer and cartilage is of interest, since other studies have shown that apoptosis is increased in RA, particularly in the synovial lining layer and cartilage (Firestein et al, 1995; Nakajima et al, 1995). NO derived through iNOS expression by proinflammatory cytokines in osteoblast is one of the possible factors to cause apoptosis of the cell (Mogi et al, 1999; 2000). Transcriptional control of osteoblast apoptosis has been reported, in which nuclear factor-kappa B (NF-κB) and/or activator protein-1 (AP-1) seem to be an important apoptotic signal pathway (Chae et al, 2000a,b).

The rat osteoblast-like, osteosarcoma cell line, ROS 17/

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ABBREVIATIONS: iNOS: inducible nitric oxide synthase; NO: nitric oxide, NF-kB, nuclear factor kappa B, AP-1: activator protein-1, PMSF: phenylmethylsulfonyl fluoride, IkB: inhibitory kappa B, PVDF: Polyvunylidene fluoride

2.8, is a well-established experimentally extensively used cell line. ROS cells not only possess a proliferative capacity that characterizes immature osteoblasts, but also express differentiated proteins wich characterize mature osteoblast; for example, alkaline phosphatase and bone morphogenic protein-2 (Wang et al, 1990; Yamaguchi et al, 1991). The rate of bone formation is largely determined by the number of osteoblasts (Parfitt, 1990), which in turn is determined by the rate of replication of progenitors and the life-span of mature cells, reflecting the timing of death by apoptosis. Because apoptosis is the fate of the majority of osteoblasts (Jilka et al, 1998), changes in the prevalence of osteoblast apoptosis should alter the rate of bone formation.

Previously, we reported that YS 49, 1-α-naphtylmethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, has diverse pharmacological actions, such as anti-thrombotic action (Yun-Choi et al, 2001), inhibition of iNOS gene expression by cytokines in RAW 264.7 cells as well as vascular smooth muscles (Kang et al, 1999a, 1999b, 2002). In the present study, we examined the effect of YS 49 on the viability and apoptosis of osteoblast cell, ROS 17/2.8, when activated with combination of pro-inflammatory stimulants,including IFN-γ, TNF-α and LPS. We found that YS 49 concentration-dependently reduced iNOS expression by inhibiting of NF-κB translocation. Furthermore, NF-κB was inhibited by inhibiting the degradation of the nuclear $\kappa B\alpha$ (I$\kappa B\alpha$) inhibitor in the cytoplasm. Finally, YS 49 was found to prevent NO-mediated apoptosis of the cell. Our results, therefore, identified a unique mechanism of action of a new synthetic isoquinoline alkaloid to exert its anti-inflammatory effects in osteoblast cells.

METHODS

Materials

Lipopolysaccharide (E. Coli; serotype 0128:B12), sulphanilamide, N-[1-naphthyl]ethyleneamine, sodium chloride, leupeptin, pepstatin A, actinomycin, and phehenylmethylsulfonylfluoride (PMSF) were from Sigma (St. Louis, MO. USA), TNF-α, IFN-γ, and iNOS antibodies were from Transduction Laboratories (Lexington, KY, USA), I$\kappa B\alpha$- and p-I$\kappa B\alpha$ antibody were from NEB., Inc, and horseradish peroxidase labeled goat anti-rabbit IgG was purchased from Jackson Immuno Research Laboratories INC (West Grove, USA). ECL Western blotting detection reagent was from Amersham (Buckinghamshire, U.K.).

Cell culture

ROS 17/2.8 cells were obtained from the American Type Culture Collection (ATTC, Rockville, MD, USA). The cells were grown in RPMI-1640 medium supplemented with 25 mM N-(2-hydroxyethyl)piperazine-N-2-ethanesulphoneic acid (HEPES), 100 U/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated fetal calf serum.

Cell stimulation

The cells were stimulated with combination of IFN-γ (10 U/ml), TNF-α (10 U/ml) and LPS (1 μg/ml) for 24 h and, when needed, YS 49 (10~100 μM) was added simultaneously or 1 h prior to cytokine mixture administration. YS 49 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 metabolites, nitrite, as described previously (Green et al, 1981; Kang et al, 1999a). At the end of incubation, 100 μl of the culture medium were mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). Absorbance at 550 nm was measured, and the nitrite concentration was determined by using a curve calibrated on sodium nitrite standards.

Assay for iNOS mRNA expression

Total RNA was extracted as described previously (Kang et al, 1999a). A 15 μg sample of total RNA per lane was subjected to electrophresis on formaldehyde conteining 1% agarose gels and transferred to nylon filters. The filters were then hybridized with a random-primed 32P-labeled iNOS cDNA probe in rapid hybridization solution (Quikhyb) at 68°C for 1 hr. The sequence of the sense primer for iNOS was 5'-TGGACCAGTATAAGGCAAGC-3' and the antisense primer was 5'-GCTCTGGATGAGCCTATATTG-3'. The hybridized filters were subsequently washed with 2 x SSC (0.3 mM sodium chloride/3 mM sodium citrate)/0.1% SDS twice for 15 min at room temperature and then washed with 0.2 x SSC/0.1% SDS at 42°C twice for 15 min. The filters were then exposed to an x-ray film.

Assay for iNOS mRNA stability

RAW 264.7 cells were treated with IFN-γ, TNF-α and LPS or IFN-γ, TNF-α and LPS for 6 h. Actinomycin D was added to the cultures (final concentration of 5 μg/ml), and the cells were harvested 0, 1, 2, 3, 4 and 5 h after the addition of actinomycin D. Total RNA was prepared and analyzed by Northern blot hybridization for iNOS mRNA as described above. Densitometry of autoradiographs was used to determine and quantify the levels of iNOS mRNA. Estimates of the relative iNOS mRNA amounts were obtained by dividing the peak densitometry area of iNOS mRNA band by the area of the GAPDH band.

Assay for iNOS protein expression

iNOS protein was analyzed by immunoblotting with the anti-iNOS antibody as described previously (Kang et al, 1999a). Briefly, the lung tissues were homogenized in a buffer containing 50 mM Tris/Cl, pH 7.5, 1 mM EDTA, 1 mM leupeptin, 1 mM pepstatin A, 0.1 mM PMSF, and 1 mM dithiothreitol, and sonicated. The homogenates were then centrifuged at 7500 x g for 15 min 4 times, and the supernatants were subjected to SDS-PAGE (7.5% gel), using the buffer system of Laemmli (1970). The separated proteins were electrophoretically transferred to PVDF membranes, and the membrane was incubated with 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 manufacturer instruction.

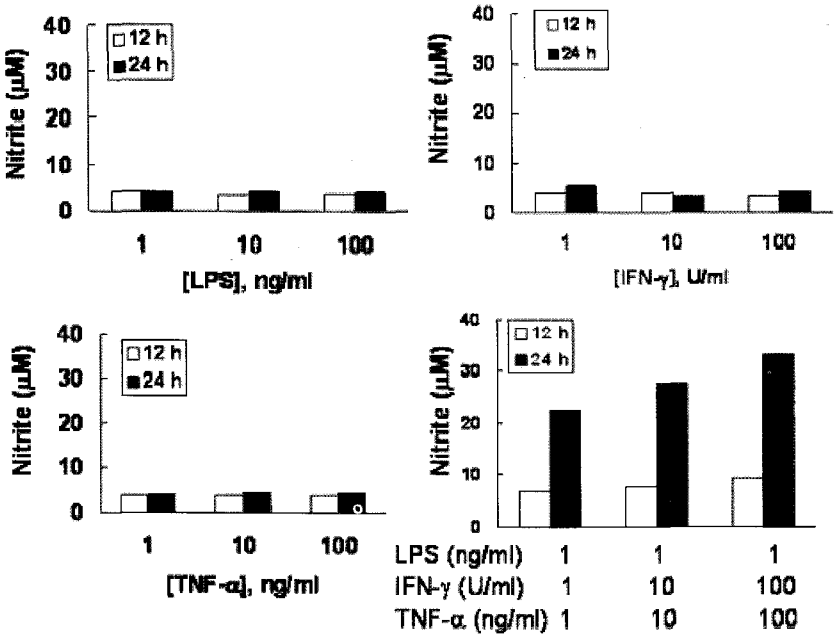

Fig. 1. Production of nitrite in ROS 17/2.8 cells depends on combination of cytokines. Cells were incubated for 12 h (opened square) or 24 h (closed square) with different combination of cytokines as indicated. Combinations of three resulted in significant accumulation of nitrite.

Western blot for I κB α and I κB α phosphorylation

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 YS 51 for 1 h before the addition of cytokines and LPS. After incubation, the cells were collected into buffer solution and sonicated. Proteins were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked for 1 hr in TTBS containing 5% nonfat dry milk and incubated for 1.5 h in primary antisera (anti-rabbit I κB α, 1 : 1000); anti-rabbit p-I κB α, 1 : 1000) containing 1% 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 : 7000. The blots were washed three times with TTBS at room temperature. The proteins (I κB α, and p-I κB α) were detected by using ECL reagents.

Genomic DNA laddering experiment

After incubation for 24 h, the cell layer was rinsed twice with 5 ml of PBS. Genomic DNA isolation was performed, as specified by the manufacturer's protocol (Promega, Madison, WI, U.S.A.). Briefly, cells were lysed with lysis buffer directly on the plate after medium was removed, and followed by 15 min incubation with RNase A. The cell lysates were treated with....to precipitate protein and spun at 13,000 g for 1 min.The supernatant was treated with isopropanol to isolate DNA. After an alcohol wash, DNA was hydrated and quantified, and 20 μg were analyzed in 1.5% agarose gel electrophoresis fractionation. DNA was visualized with ethidium bromide.

Statistical evaluations

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

RESULTS

Production of NO by combination treatment of TNF-α, IFN-γ and LPS

In ROS 17/2.8 cells, each cytokine or LPS separately did not produce NO (Fig. 1). However, NO production was increased only by combinations of three, which was concentration-and incubation time-dependent. For example, the amount of NO, as measured as nitrite was, 6±0.4 μM in control media, which was increased to 11±3.5 μM and 23±3.5 μM by combination of LPS, IFN-γ and TNF-α for 12 h and 24 h incubation, respectively. The NO produced was dependent concentration on the of cytokines added; for 24 h incubation, it was 23±3.5 μM in LPS (1 ng/ml), IFN-γ (10 U/ml) and TNF-α (10 U/ml), which was increased to 44±3.5 μM in LPS (1 ng/ml), IFN-γ (100 U/ml) and TNF-α (100 U/ml). Consequently, the data represented in the present study are those of combinations of LPS (1 ng/ml), IFN-γ (10 U/ml) and TNF-α (10 U/ml).

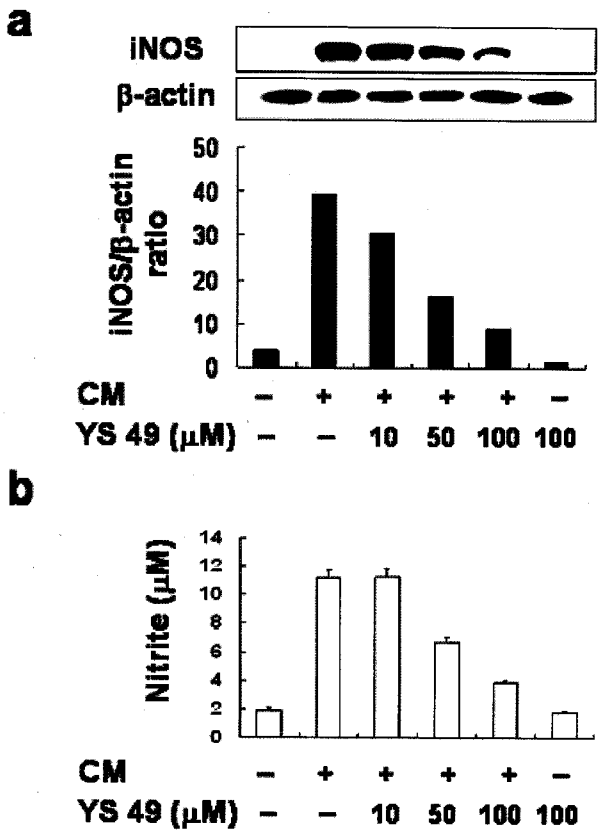

Fig. 2. A representative Western blot image of iNOS protein and nitrite production by YS 49 in ROS 17/2.8 cells activated with CM. (a) The upregulated expression of iNOS protein by CM was concentration-dependently reduced by YS 49 (b) YS 49 concentration-dependently reduced NO production. Data represent mean ±s.e.m. of four separate experiments.

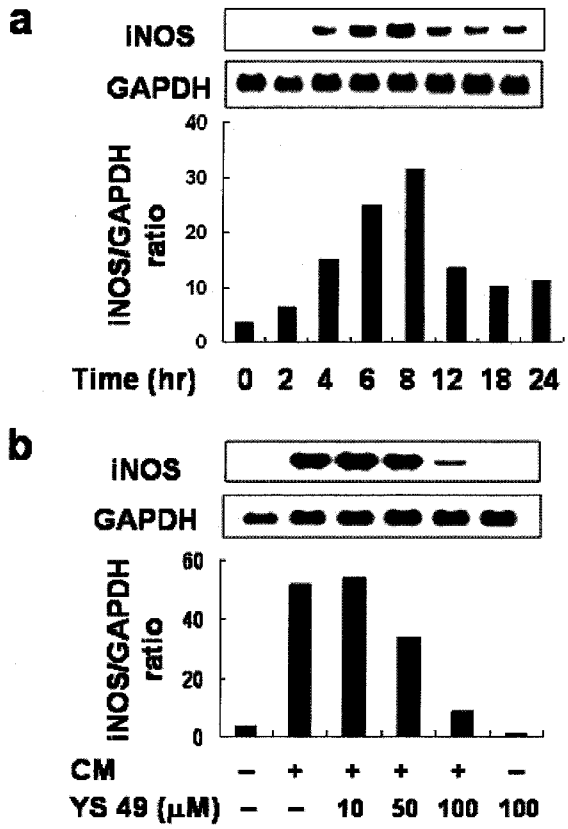

Fig. 3. Time course of the expression pattern of iNOS mRNA by CM and effects of YS 49 on iNOS mRNA expression. (a) iNOS mRNA was maximally expressed after 8 h incubation of CM and it still remained after 24 h. (b) Concentration-dependent inhibition of the of iNOS mRNA expression by YS 49.

Effects of YS 49 on production of NO and iNOS protein expression

Fig. 2 shows the inhibitory effect of YS 49 on the production of NO and iNOS protein expression. As shown in Fig. 2a, as the concentration of YS 49 increased, the iNOS expression diminished, resulting in decreased NO production (Fig. 2b). The concentration of YS 49 50% to inhibit of NO production (IC_{50}) was 56.4±7 μM.

Effects of YS 49 on iNOS mRNA expression and stability

As shown in Fig. 3a, Northern analysis indicated that the cytokine mixture time-dependly, induced iNOS mRNA time-dependently, in which the message appeared from 4 h incubation, reaching maximum at 8 h, thereafter decreasing as time lapsed, but it still remained until 24 h. When the effect of YS 49 on iNOS mRNA expression was exameined, the messages were significantly diminished by the presence of YS 49, which was concentration-dependent (Fig. 3b). In order to examine whether this downregulation of iNOS mRNA was related with its stability, we examined the half life of the iNOS mRNA by using actinomycin D. As shown in Fig. 4, the slope of mRNA degradation was almost the same between the cytokine mixtures - and cytokine plus YS 49, treatment thur indicating that YS 49 did not interference with its stability.

Effects of YS 49 on NF-κB translocation and IκBα phosphorylation by the mixture of cytokines and LPS

In order to understand the possible mechanism(s) by which YS 49 inhibited activation of NF-κB, an effect of YS 49 on IκBα phosphorylation was investigated. As shown in Fig. 5a, YS 49 concentration-dependently inhibited the translocation of NF-κB (p65) from cytoplasm to nucleus. Next a question of whether, YS 49 inhibited translocation of NF-κB was due to inhibition of degradation of IκBα by prevention of IκBα phosphorylation was investigated. As shown in Fig. 5b, YS 49 concentration-dependently inhibited the phosphorylation of IκBα, thereby resulting in reduction of degradation of IκBα. Consequently, the activation of NF-κB was prevented by YS 49.

Effects of YS 49 on NO-mediated apoptosis

Finally, we wanted to find out whether YS 49 prevented

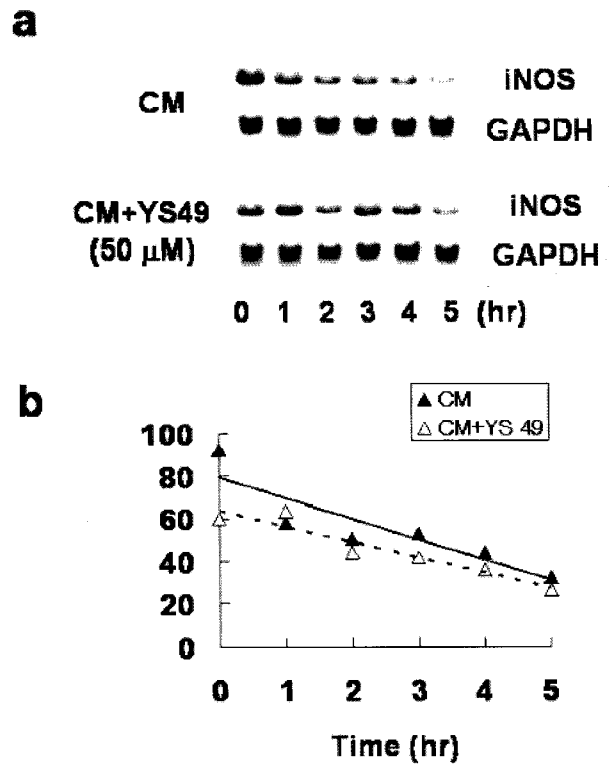

Fig. 4. Effect of YS 49 on iNOS mRNA stability. Expression of iNOS mRNA was investigated in the presence of actinomycin. (a) Time course of the expression of iNOS mRNA by CM (upper) or CM with YS 49 (lower) in the presence of actinomycin. (b) Comparison of mRNA decay slope between CM and CM with YS 49.

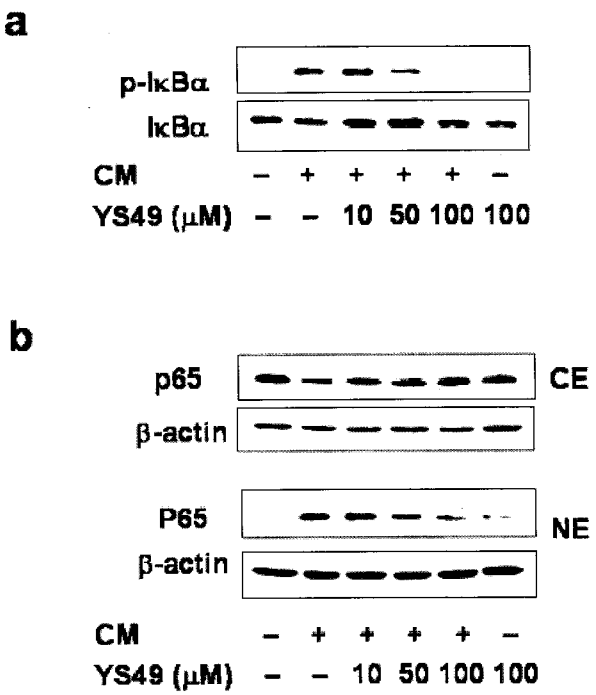

Fig. 5. Effect of YS 49 on NF-κB activation. (a) YS 49 concentration-dependently reduced NF-κB complex. (b) The inhibitory action of YS 49 on NF-κB activation was due to inhibition of p 65 translocationan and IkBα phosphorylation. YS 49 concentration-dependently retained p65 in cytosol fraction (CE, cytosol extracted fraction), therefore little appears in nuclear extracted fraction (NE) as shown in upper panel. (b) This inhibition of translocation was due to inhibition of IκBα phosphorylation by YS 49.

apoptotic cell death from excess of NO. To provide such environment, we used high concentration of sodium nitroprusside (SNP), NO donor. In seen Fig. 6, DNA-strand break was clearly shown in those cells activated with SNP, which was decreased by 100 μM YS 49,thus indicating that YS 49 reduced apoptotic cell death due to excess of NO.

DISCUSSION

Bone metabolism is closely regulated by hormones and cytokines, which affect both bone resorption and deposition. Osteoblasts, typically located on bone-lining surfaces, are physically positioned to significantly influence bone resorption. 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 rheumatoid arthritis (RA). Proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α can as powerful local stimulatory signals for bone resorption (Hukkanen et al, 1995). 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. IL-1β and TNF-α may also inhibit osteoblast activity, suggesting a mechanism for uncoupling of bone resorption and deposition during inflammation. In addition to IL-1β and TNF-α, LPS has also been shown to exert direct effects on bone remodeling, as well as to stimulate the production of other cytokines (e.g., IL-1β, TNF-α, and IL-6) by osteoblasts (Tom et al, 1998). In fact, inflammatory conditions such as RA as well as osteoarthritis (OA), which are characterized by local osteolysis, are associated with activation of inducible nitric oxide synthase (iNOS) (Grabowski et al, 1997; Boileau et al, 2002). Activation of the iNOS by inflammation has been related with apoptotic cell death in many cells including osteoblast (Armour et al, 2001). These findings indicate that cytokines could modulate the life span of osteoblasts via apoptosis, thus regulating bone metabolism in certain pathologic conditions such as periarticular osteoporosis found in patients with RA.

In this study, we demonstrated that YS 49, a synthetic isoquinoline alkaloid, inhibited iNOS induction in ROS 17/2.8 osteoblast cells activated with pro-inflammatory cytokines and LPS. LPS and/or cytokines have been shown to induce the expression of the iNOS isoform (Forstermann et al, 1995) in many cell types, including osteoblasts (Fox and Chow, 1998). We found that single cytokines had little or no stimulatory effect on NO production in ROS 17/2.8 cells, however. The combination of IFN-γ, TNF-α, and LPS resulted in a dramatic stimulation of NO production in a highly reproducible manner. In murine iNOS gene, cytokine responsive elements for binding the transcriptional

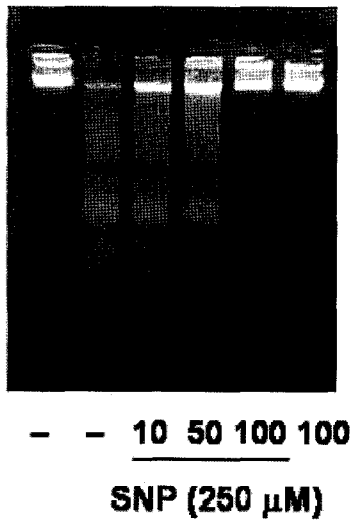

Fig. 6. Effects of YS 49 on DNA strand break induced by sodium niotroprusside (SNP). High concentration of SNP (250 μM) produced DNA ladders which was reduced by YS 49.

factors such as activating protein-1 (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). The concentration-dependent reduction of NO production by YS 49 can, therefore, be explained as the result of inhibition of NF-κB activation by the prevention of phosphorylation of IκBα, which hinders the translocation of activated NF-κB to nucleus from cytosol to initiate iNOS gene transcription. It is quite reasonable to speculate that YS 49 may affect NF-κB inducing kinase or IκBα kinase activity, thus inhibiting the phosphorylation of IκBα.

However further investigation is needed to prove this possibility.

That YS 49 interferes iNOS gene expression at the transcriptional stage is further evidenced by iNOS mRNA stability experiment, in which YS 49 did not affect the half-life of iNOS mRNA in the presence of actinomycin D. On the other hand, NO has been reported to markedly inhibit osteoblast cell proliferation (Ralston, 1994; Hukkanen, 1995). Furthermore, has been known to be related with the pathogenesis of arthritis, evidenced from studies showing that high levels of nitrite/nitrate are present in serum and in the synovial fluid of arthritis patients, and that the mRNA and protein of iNOS have been detected in the synovial tissues of both osteoarthritis and RA patients (Wang et al, 1990; Yamaguchi et al, 1991). In the present study, YS 49 significantly inhibited DNA fragmentation induced by excess amount of NO, sodium nitroprusside.

NO derived from iNOS expression by proinflammatory cytokines in osteoblast is one of the possible factors to cause apoptosis of the cell (Mogi et al, 1999, 2000). Transcriptional control of osteoblast apoptosis has been reported, in which nuclear factor-kappa B (NF-κB) and/or activator protein-1 (AP-1) seems to be an important apoptotic signal pathway (Kitajima et al, 1996; Chae et al, 2000a, 2000b). Furthermore, NO acting as a paracrine factor is also recognized as an important factor in bone remodeling, such as, osteoblast apoptosis (Armour et al, 2001), cartilage loss and degradation in arthritis joint as apoptosis of chondrocytes (Pelletier et al, 2001). NO has several effects which may be relevant to pathogenesis of tissue damage in RA, including stimulation of blood flow, inhibition of matrix production by chondrocytes (Taskiran et al, 1994), activation of metalloproteinases (Murrell et al, 1995), modulation of immune response (Wei et al, 1995), suppression of osteoblast activity (Ralston et al, 1994) and enhancement of cytokine-induced osteoclastic bone resorption (Ralston et al, 1996). Despite being a transformed cell line, the ROS 17/2.8 cells are able to undergo NO-induced apoptosis. It is proven that ROS 17/2.8 cells are vulnerable to apoptotic cell death with modification of serum restriction (Ihbe et al, 1998). 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 wich defines the osteoblast-osteocyte transition (Wetterwald et al, 1996), and can serve as a good model for investigating the mechanisms of osteoblastic apoptosis (Ihbe et al, 1998).

The rate of bone formation is largely determined by the number of osteoblasts (Yudoh et al, 2001), which is in turn determined by the rate of replication of progenitors and the life-span of mature cells, reflecting the timing of death by apoptosis. Because 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 numbers of functional osteoblasts, thereby prolonging the bone formation period. The mechanisms responsible for apoptosis in the rheumatoid joint remain unclear, although previous studies have shown that expression of Fas antigen is increased in RA synoviocytes (Nakajima et al, 1995; Sioud et al, 1998), and that Fas antibody can stimulate the apoptotic death of synoviocytes in vitro (Nakajima et al, 1995). Althought our study did not address the apoptosis for regulation of disease activity in RA, it is higly likely that activation of the iNOS pathway promotes apoptosis within the joint. Clarification of interrelationships between iNOS activation, apoptosis and disease activity in RA will, however, require further studies with NOS inhibitors *in vivo*.

In summary and conclusion, we provided evidence in the present study that the ability of YS 49 to suppress iNOS gene expression was responsible for prevention of apoptosis of ROS 17/2.8 induced by large quantity of NO. Therefore, YS 49 is expected to be beneficial against NO-mediated inflammatory bone disorders such as arthritis and osteoporosis.

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