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Effects of nitric oxide from exogenous nitric oxide donors on osteoblastic metabolism

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Abstract

We examined the effects of nitric oxide (NO) on the differentiation and mineralization of newborn rat calvarial osteoblastic cells (ROB cells) using exogenous NO donors, sodium nitroprusside, 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine (NOC-7) and 2,2′-(hydroxynitrosoydrazino)bis-ethanamine (NOC-18). Sodium nitroprusside and NOC-7 dose-dependently enhanced the rate of production of intracellular cGMP in ROB cells and the rat clonal osteogenic cell line ROB-C26. We used NOC (NOC is the trade name for NO complex manufactured by Dojindo, Kumamoto, Japan) as an NO donor in our experiments because sodium nitroprusside exhibited a marked cytotoxicity. Northern blot analysis revealed that the level of mRNA for osteocalcin, one of the osteoblastic differentiation markers, was enhanced in the ROB cells, which was continuously treated by NOC-18. NOC-18, however, did not affect the level of mRNA for alkaline phosphatase and the activity of alkaline phosphatase. Both the number and the total area of mineralized nodules that are a model of in vitro bone formation were shown to be increased by 10^{-5} M NOC-18. Our data suggest that NO might act as a local regulator of the metabolism of osteoblastic cells.

Keywords: Nitric oxide (NO); Nitric oxide (NO) donor; cGMP; Soluble guanylate cyclase; Osteoblast

1. Introduction

Nitric oxide (NO) is synthesized from L-arginine by nitric oxide synthase (NO synthase) that consists of three isoforms, inducible-type NO synthase and two constitutive-type NO synthases (Moncada et al., 1991). NO is also formed experimentally from synthetic NO donors, such as sodium nitroprusside or zwitterionic polyamine/NO (e.g., NOC). Sodium nitroprusside spontaneously releases NO by an as yet unknown mechanism (Feelisch, 1991). Sodium nitroprusside, however, is likely to have a cytotoxic effect, as well as additional effects on other regulatory systems unrelated to the generation of NO; the neuroprotective properties of sodium nitroprusside on glutamate-induced neurotoxicity are determined by the ferrocyanide portion of the sodium nitroprusside molecule (Kiedrowski et al., 1991; Dawson et al., 1991). In contrast, NOC is an ideal NO donor because NOC generates NO spontaneously under time control; the half-life of NOC-7 and NOC-18 is 5 min and 21 h, respectively. No additional function and no toxicity of NOC have been reported (Hrabie et al., 1993). The appropriate choice of endogenous NO donors is important when studying the formation and function of NO. NO formed in cells activates soluble guanylate cyclase, with a resultant increase in the rate of intracellular production of cGMP, and regulates a wide range of functions throughout the body. In osteoblastic cells, the expression of both types of NO synthase has been reported (Helfrich et al., 1997; Hikiji et al., 1997; Riancho et al., 1995; Lowik et al., 1994; Damoulis and Hauschka, 1994), but little is known about the effects of NO on the differentiation of osteoblastic cells.

In previous studies, we have proposed that cGMP produced in response to natriuretic peptides (Inoue et al., 1996; Hagiwara et al., 1996) or 8-bromo-cGMP (Inoue et al., 1995) promotes the differentiation and mineralization of osteoblastic cells. Therefore, we have postulated that

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NO might be involved in osteoblastic metabolism through the production of cGMP. The purpose of our present study is to elucidate the contribution of NO to osteoblastic metabolism. To investigate this issue, we used sodium nitroprusside and NOC as NO donors and newborn rat calvarial osteoblastic cells (ROB cells) and rat calvarial osteogenic cell line ROB-C26 (Yamaguchi and Kahn, 1991a) as a culture system for the osteoblastic cells. ROB cells are heterogenous. However, it is well known that this culture system is useful for studying bone formation in vitro (Antosz et al., 1989; Harris et al., 1994; Hagiwara et al., 1996). We demonstrated that NO might contribute to the regulation of osteoblastic metabolism.

2. Materials and methods

2.1. Isolation and culture of cells

ROB cells were isolated enzymatically from the calvariae of newborn Wistar rats. The constituents of the enzyme mixture and the sequential digestion procedure for the isolation of rat calvarial cells have been described previously (Hagiwara et al., 1996). The cells were replated in 12-well plates (3.8 cm^2/well) at a density of 1×10^4 cells/cm^2 and grown in α-modified minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid. During subculture, the medium was replaced every 4 days and reagents were added every 2 days.

Rat calvarial osteogenic cell line, ROB-C26, was plated in 12-well plates (3.8 cm^2/well) at a density of 1×10^4 cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid. During subculture, the medium with reagents were replaced every 3 days.

2.2. Measurements of the accumulation of cGMP

Cells, grown in 12-well plates for 4 days or 14 days, were incubated with serum-free α-MEM supplemented with 0.5 mM of 3-isobutyl-1-methylxanthine, diesterase inhibitor, at 37°C for 15 min after being washed with serum-free α-MEM. Osteoblastic cells were subsequently incubated at 37°C for 1 h with NO donors. After incubation, the amount of cGMP in each media was measured with a radioimmunoassay kit from Yamasa (Chiba, Japan).

2.3. Northern blot analysis

RNA was extracted from cultured osteoblastic cells by the acid guanidinium–phenol–chloroform method (Chomczynski and Sacchi, 1987). Total RNA (20 μg) was subjected to electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and was then transferred to a MagnaGraph nylon membrane (Micron Separations, Westborough, MA, USA). After baking the membrane, the RNA on the membrane was allowed to hybridize overnight with cDNAs for alkaline phosphatase, osteocalcin (Yamaguchi et al., 1991b), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 42°C in 50% formamide containing 6 × sodium chloride/sodium phosphate/EDTA (SSPE) solution (1 × SSPE is 0.15 M NaCl, 15 mM NaH_2PO_4, pH 7.0, 1 mM EDTA), 2 × Denhardt's solution (0.1% each of bovine serum albumin, polyvinylpyrrolidone and Ficoll), 1% sodium dodecyl sulfate (SDS) and 100 μg/ml herring sperm DNA. Each cDNA probe was radiolabeled with a Ready-to-Go (Pharmacia, Uppsala, Sweden). The membrane was washed twice in 1 × sodium chloride/sodium citrate (SSC) solution (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at room temperature for 5 min each, and twice in 1 × SSC containing 0.1% SDS at 55°C for 1 h each. It was then exposed to an imaging plate for 4 h. The plate was analyzed with a Bioimage Analyzer (BAS 2000; Fuji Film, Tokyo, Japan).

2.4. Assay of alkaline phosphatase activity

Osteoblastic cells were subcultured in α-MEM containing 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid and NO donors. The cells were washed twice with 50 mM Tris–HCl, pH 7.2 and sonicated in 1 ml of 50 mM Tris–HCl, pH 7.2, containing 0.1% Triton X-100 and 2 mM $MgCl_2$ for 15 s with a sonicator (Ultrasonic Disruptor UD-201; Tomy, Tokyo, Japan). The activity of alkaline phosphatase in the sonicate was determined by an established technique with *p*-nitrophenyl phosphate as the substrate (Lowry et al., 1954). Protein concentrations were determined with a BCA protein assay reagent (Pierce Chemical, Rockford, IL, USA).

2.5. Quantitation of calcium

The mineralized nodules were washed twice with phosphate-buffered saline (PBS) and incubated with 1 ml of 2 N HCl overnight with gently shaking. The calcium ions were quantitated by the *o*-cresolphthalein complexone method with a Calcium C-Test Wako (Wako, Osaka, Japan) (Hagiwara et al., 1996).

2.6. von Kossa staining

Osteoblastic cells in 12-well plates (3.8 cm^2/well) were fixed with 10% formaldehyde for 30 min and washed three times with 10 mM Tris–HCl, pH 7.2. The fixed cells were incubated with 5% silver nitrate for 5 min in sunlight, washed twice with H_2O, and then treated with 5% sodium thiosulfate (Hagiwara et al., 1996). Mineralized nodules were assessed with respect to their number, and the total area of nodules using an automated imaging system, which

consisted of a BH microscope (Olympus), a camera (CCD/ICD-740, Olympus) and Mac SCOPE program (Mitani, Fukui, Japan).

2.7. Drugs

Sodium nitroprusside, $Na_2[Fe(NO)CN_5]$, was purchased from Sigma, NOC-7 (half life of 5 min), 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine and NOC-18 (half-life of 21 h), 2,2′-(hydroxynitrosohydrazino)bis-ethanamine were from Dojindo, Kumamoto, Japan. ^{32}P-labeled nucleotides were obtained from Amersham Life Science, Buckinghamshire, UK, α-MEM, fetal bovine serum and penicillin/streptomycin antibiotic mixture were obtained from Life Technologies, Grand Island, NY, USA.

3. Results

3.1. Production of cGMP by NO donors in ROB cells and ROB-C26

We measured, on day 4, the production of intracellular cGMP by the addition of NO donors such as sodium nitroprusside and NOC-7 to the culture medium of ROB cells (Fig. 1A) and of rat clonal osteogenic cell ROB-C26 (Fig. 1B). Both NO donors dose-dependently enhance the rate of production of cGMP through the soluble guanylate cyclase in the cells. NOC-7 was more effective in the production of cGMP than of sodium nitroprusside. The production of cGMP by 10^{-5} M NOC-7 were 76.4 ± 5.5 pmol/10^6 cells in ROB cells, and 49.8 ± 1.9 pmol/10^6 cells in ROB-C26 cells. On day 14, the production of cGMP by 10^{-5} M sodium nitroprusside and NOC-7 were 10.3 ± 0.7 pmol/10^6 cells and 24.3 ± 2.7 pmol/10^6 cells, respectively, in ROB cells. These observations indicate that soluble guanylate cyclase is present in osteoblastic cells during the experiments.

Sodium nitroprusside has been reported to be cytotoxic, due either to its degradation products or the sodium nitroprusside molecule itself. We also observed the sodium nitroprusside-induced damage of ROB cells (data not shown). However, NOC-18 appeared to cause no morphological changes or cell death under microscopic observation. Therefore, we used NOC-18 as an NO donor in our experiments with osteoblasts.

3.2. Regulation of osteoblastic differentiation by NO donors

We examined the effects of NO formed from NOC-18 on the differentiation and mineralization (formation of mineralized nodules) of ROB cells. We first used a Northern blot analysis to investigate the level of mRNAs for osteoblastic differentiation marker proteins, such as alkaline phosphatase and osteocalcin, in ROB cells treated with NOC-18 for the indicated period (Fig. 2). The continuous treatment of ROB cells by NOC-18 resulted in a marked increase (3.5-fold) in the level of mRNA for osteocalcin, while the level of mRNA for alkaline phosphatase was similar to that of the control. There was no change in the activity of alkaline phosphatase on the ninth day of the continuous culture of ROB cells in the presence and the absence (control) of NOC-18 (data not shown). To examine the effect of NOC-18 on the mineralization by osteoblastic cells, we measured the accumulation of calcium in

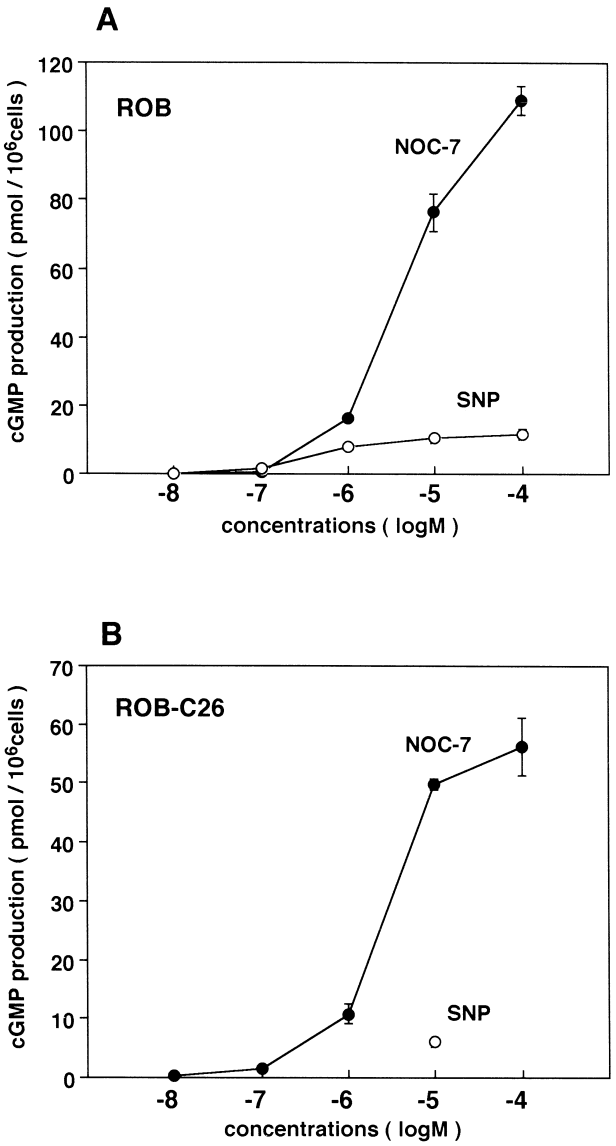

Fig. 1. The production of cGMP by NO donors in osteoblastic cells. ROB cells (A) and clonal osteogenic cell ROB-C26 (B), grown in 12-well plates (3.8 cm^2/well) for 4 days, were exposed to sodium nitroprusside or NOC-7 at various concentrations for 1 h at 37°C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine, and levels of cGMP were determined as described in Section 2. Each value is the mean ± S.E. of results of three or four wells. Results are representative of experiments that were each performed three times with similar results.

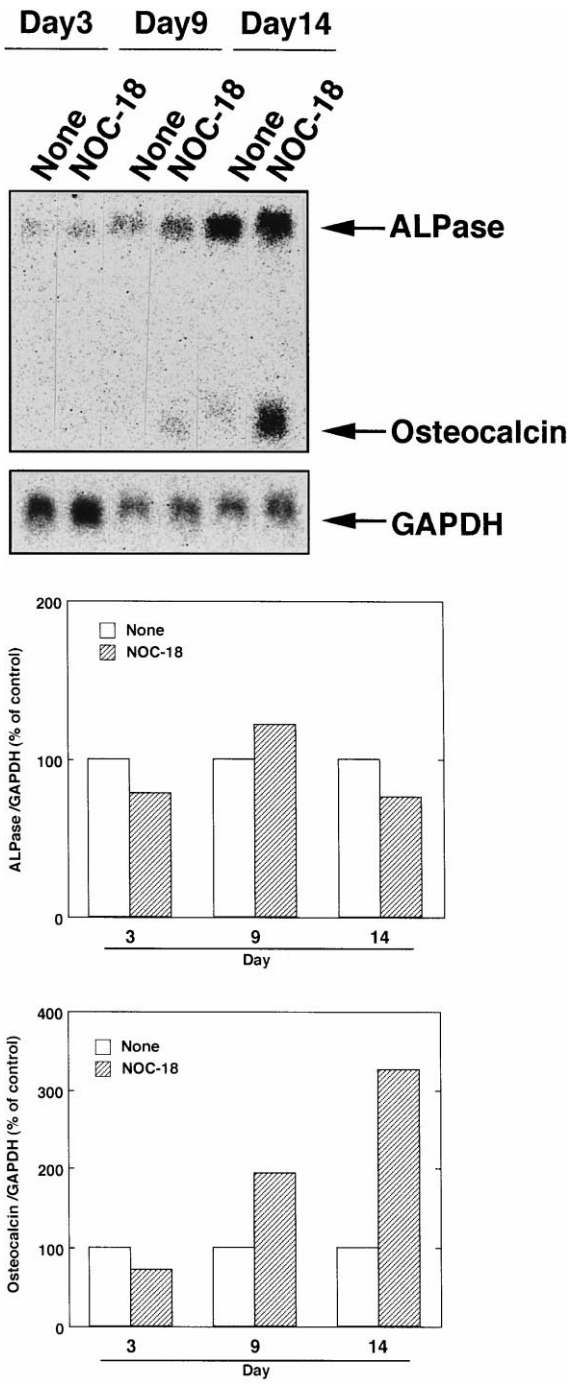

Fig. 2. Northern blot analysis of mRNAs for osteoblastic differentiation markers in cells treated by NOC-18. Total RNA was isolated from ROB cells after treatment with 10 μM NOC-14 for 3, 9 and 14 days. Twenty μg of total RNA were subjected to electrophoresis in an agarose gel, and were allowed to hybridize with ^{32}P-labeled cDNA for rat alkaline phosphatase, osteocalcin, or GAPDH, as described in Section 2. There were no appreciable changes in the level of mRNA for GAPDH in cells assayed similarly on the same respective days. The relative osteocalcin or alkaline phosphatase mRNA concentration was analyzed with a Fuji Film Biomage Analyzer and normalized with the GAPDH level. The ratio of osteocalcin/GAPDH or alkaline phosphatase/GAPDH for the control culture at day 3 was defined as 100%.

the cell and matrix layer. The accumulation of calcium in ROB cells treated by NOC-18 for 14 days was slightly increased (data not shown). Fig. 3 shows the results of von

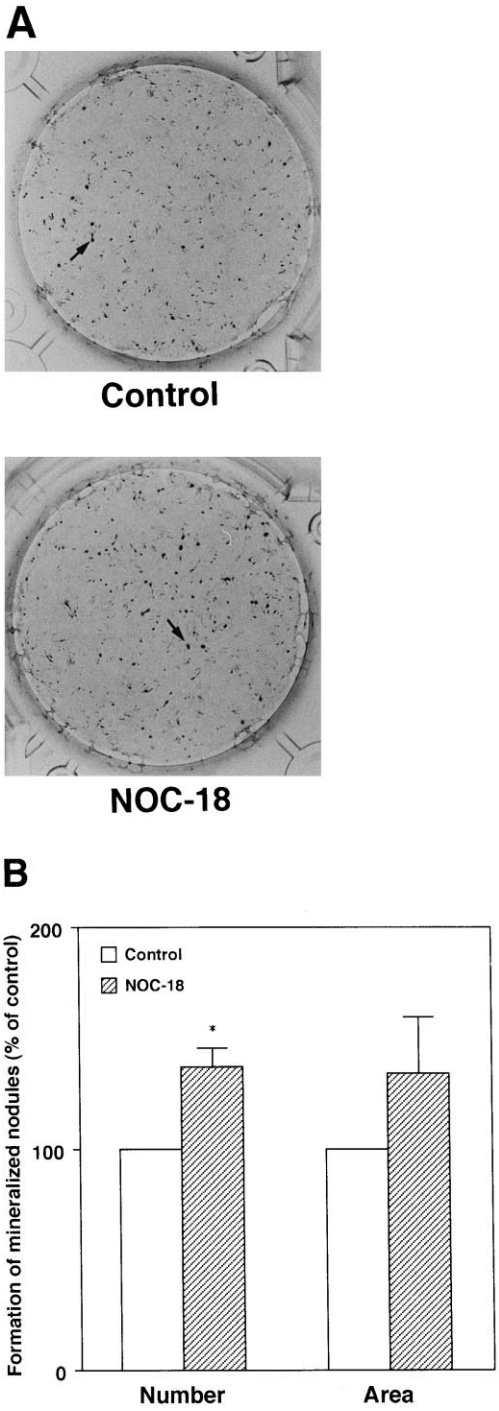

Fig. 3. Formation of mineralized nodules in cultures of ROB cells treated with NOC-18. Cells in 6-well plates (9.4 cm^2/well) were cultured for 11 days with α-MEM containing 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml L-ascorbic acid and 10^{-5} M NOC-18. Mineralized nodules were subjected to von Kossa staining as described in Section 2. Phase-contrast photomicrographs (A). Numbers and areas of mineralized nodules were determined with the Mac SCOPE program (B). Arrows, mineralized nodules stained by von Kossa staining. Data are means $\pm$ S.E. of results 3 wells. $* P < 0.02$ vs. control.

Kossa staining of mineralized nodules that had been formed by ROB cells during cultivation for 11 days in medium supplemented with 10^{-5} M NOC-18. In the presence of NOC-18, about 130% of the control number and the total area of mineralized nodules were formed. These results suggest that NO might promote the differentiation and mineralization process of osteoblastic cells.

4. Discussion

The endogenous formation of NO by NO synthase and the subsequent stimulation of soluble guanylate cyclase is an important pathway for cell function and cell communication. However, little is known about the effects of NO on osteoblastic metabolism, although the inducible-NO synthase (Helfrich et al., 1997; Hikiji et al., 1997; Riancho et al., 1995; Lowik et al., 1994; Damoulis and Hauschka, 1994) and constitutive NO synthase (Helfrich et al., 1997; Riancho et al., 1995) have been identified in various types of osteoblastic cells. In the present paper, we have shown that NO stimulated the rate of cGMP production in rat calvarial osteoblast-like cells (ROB cells) and rat clonal osteogenic cell ROB-C26. These results demonstrate the existence of soluble guanylate cyclase in those cells. Moreover, we have also shown that NO might be involved in the differentiation and mineralization process of osteoblastic cells via NO itself and/or a cGMP-mediated mechanism.

Sodium nitroprusside spontaneously releases NO in a nonlinear manner, but the mechanism for this NO release still remains obscure. The relatively small amounts of NO released from sodium nitroprusside in vitro are not sufficient to account for its marked enzyme-activating and dilatory potency (Feelisch, 1991). Cyanide released during decomposition may be toxic for cells. In fact, we have confirmed the toxicity of sodium nitroprusside for osteoblastic cells. In contrast to sodium nitroprusside, it is easy for a series of NOC analogs to control in time the release of NO under neutral conditions. We have shown that NOC produces cGMP more effectively than sodium nitroprusside in osteoblastic cells (Fig. 1). NOC analogs had little toxicity in osteoblastic cells (data not shown). These observation suggest that the choice of NOC as an exogenous NO donor is good for studying the formation and function of NO in osteoblastic cells.

A model demonstration of the relationship between proliferation and differentiation during the development of ROB cells has been provided, divided into three distinct processes: (1) proliferation, (2) maturation of the extracellular matrix and (3) mineralization. ROB cells express, during its developmental sequence, genes associated with differentiation, such as type-I collagen for the proliferative period, alkaline phosphatase and osteopontin for the extracellular matrix maturation period, and osteocalcin for the mineralization period (Stein et al., 1990). In the present study, NO stimulated the level of mRNA expression for osteocalcin, a marker protein of osteoblastic maturation, and the accumulation of calcium in osteoblastic cells. These results suggest that NO itself or cGMP produced by NO may promote osteoblastic metabolism. The cAMP response element are on the promoter regions of the gene for osteocalcin (Lian et al., 1989). Recently, a cGMP/cGMP-dependent protein kinase signal has been reported to activate the cAMP response element independently of cAMP/cAMP-dependent protein kinase (Gudi et al., 1996). NO may enhance the expression of the gene for osteocalcin through the cAMP response element activated by cGMP. NO had no effect on the expression of alkaline phosphatase. We have previously reported that natriuretic peptides, which are endogenous activators of membrane-bound guanylate cyclases, enhance the level of mRNA for alkaline phosphatase in ROB cells (Hagiwara et al., 1996) and MC3T3-E1 (Inoue et al., 1996). The differences in the effects between NO and the natriuretic peptides on the expression of alkaline phosphatase may reflect the additional effects of NO itself in osteoblastic cells.

The present study suggests that cGMP produced by soluble guanylate cyclase in response to NO might accelerate the differentiation and mineralization process in osteoblastic cells. We have used exogenous NO donors as NO sources in this study. As it has been reported that a variety of cytokines induce the production of NO through inducible-NO synthase in osteoblastic cells (Helfrich et al., 1997; Hikiji et al., 1997; Riancho et al., 1995; Lowik et al., 1994; Damoulis and Hauschka, 1994), NO may play a physiological role in the regulation of osteoblastic activity. There are two primary pathways for the production of cGMP: the activation of soluble guanylate cyclase by NO, and the activation of membrane-bound guanylate cyclases, such as A-type natriuretic peptide receptor and B-type natriuretic peptide receptor, by natriuretic peptides (Hagiwara et al., 1995). We have reported that 8-Br-cGMP (Inoue et al., 1995) or natriuretic peptides (Hagiwara et al., 1996; Inoue et al., 1996) promotes differentiation and mineralization, the formation of mineralized nodules, in osteoblastic cells. Our results suggest that the cGMP signal in osteoblastic cells stimulate the differentiation and mineralization. There are some reports of the inhibition of the differentiation and bone-resorption activity by NO in osteoclasts (Holliday et al., 1997; Kasten et al., 1994; Maclntyre et al., 1991). However, a low concentration of NO has been reported to increase the generation of osteoclasts, although NO inhibits osteoclast generation in a high concentration (Ralston and Grabowski, 1996; Chae et al., 1997). In osteoclast, the different effects of NO may be observed by difference of concentration of NO and of culture system. Under the circumstances, the NO system may be involved in the prevention of osteoporosis through the activation of osteoblasts and the inhibition of osteoclasts. Our observation may also imply a future therapeutic use of NO-generating agents for the patients with osteoporosis.

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