ELSEVIER

European Journal of Pharmacology 391 (2000) 225–231

ejp

www.elsevier.nl/locate/ejphar

Nitric oxide accelerates the ascorbic acid-induced osteoblastic differentiation of mouse stromal ST2 cells by stimulating the production of prostaglandin E_2

Atsushi Koyama, Eri Otsuka, Atsuto Inoue, Shigehisa Hirose, Hiromi Hagiwara *

Research Center for Experimental Biology, Tokyo Institute of Technology, Yokohama 226-8501, Japan

Received 27 September 1999; received in revised form 24 January 2000; accepted 28 January 2000

Abstract

Nitric oxide (NO) promoted the differentiation of clonal stromal cells (ST2 cells) derived from mouse bone marrow to osteoblast-like cells. The level of expression of mRNA for osteocalcin, a marker of osteoblastic differentiation, and the formation of mineralized nodules, increased in ST2 cells treated with a donor of NO. We used the reverse transcriptase-polymerase chain reaction (RT-PCR) to identify the subtypes of NO synthase that were expressed in the ST2 cells and we detected the expression of an inducible NO synthase gene in response to tumor necrosis factor-α (TNF-α). In various types of cell, NO induces the synthesis of prostaglandin E_2 and cGMP, which are known as regulators of osteoblastic differentiation, by activating cyclooxygenases and soluble guanylate cyclase, respectively. Prostaglandin E_2 was generated in response to NO in ST2 cells, however, no synthesis of cGMP in response to NO was detected. Two inhibitors of cyclooxygenase-2, *N*-[4-nitro-2-phenoxyphenyl]-methanesulfonamide (nimesulide) and 1-(4-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid (indomethacin), inhibited the formation of mineralized nodules by ST2 cells. Our observations suggest that NO might promote osteoblastic differentiation of ST2 cells by stimulating the production of prostaglandin E_2.

Keywords: Nitric oxide (NO); Stromal cell; Osteoblast-like cell; Mineralization; Differentiation

1. Introduction

A clonal line of stromal cells (ST2 cells) were isolated from the bone marrow of a Balb/c mouse and have characteristics typical of preadipocytes (Ogawa et al., 1988). ST2 cells have the potential to differentiate into osteoblast-like cells (Yamaguchi et al., 1996; Natsume et al., 1997) but they do not exhibit features typical of the osteoblastic phenotype under normal culture conditions. ST2 cells differentiate into osteoblast-like cells when bone morphogenetic proteins (Yamaguchi et al., 1996; Natsume et al., 1997) are added to the culture medium. Bone morphogenetic proteins induce the expression of mRNA for core binding factor a1 (Cbfa1), which acts as an activator of transcription in osteoblastic differentiation (Chen et al., 1998). Ascorbic acid also induces the osteoblastic differentiation of ST2 cells (Otsuka et al., 1999). Available evidence suggests that ascorbic acid might act via the activation of type I collagen/$\alpha_2\beta_1$-integrin pathways and potentiate signals due to bone morphogenetic proteins (Otsuka et al., 1999).

Nitric oxide (NO) regulates many biological functions in nervous, vascular and immune systems (Moncada et al., 1991). It is synthesized from L-arginine by NO synthase (NOS), which exists as three isoforms, inducible-type NOS (iNOS) and two constitutive-type NOS, namely, endothelial NOS (eNOS) and neuronal NOS (nNOS) (Moncada et al., 1991). The expression of both the inducible and constitutive-expressed NOS has been recognized in osteoblastic cells (Damoulis and Hauschka, 1994; Lowik et al., 1994; Helfrich et al., 1997; Hikiji et al., 1997) but the expression of NOS has not been examined in stromal ST2 cells. NO formed in cells is known to activate two classes of hemoproteins, cyclooxygenases and soluble guanylate cyclases, with resultant increases in the rates of intracellular production of prostaglandins and cGMP, respectively. NO appears to be an important regulator of the formation and resorption of bone (Ralston and Grabowski, 1996; Chae et

* Corresponding author. Tel.: +81-45-924-5830; fax: +81-45-924-5832.

E-mail address: hhagiwar@bio.titech.ac.jp (H. Hagiwara).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00100-X

al., 1997; Sato et al., 1998). However, there have been no reports, to our knowledge, on the effects of NO on the osteoblastic differentiation of stromal cells. In this study, we found that NO potentiated the osteoblastic differentiation of ST2 cells and that this effect might have been mediated by the prostaglandin E_2.

2. Materials and methods

2.1. Cell culture

Mouse bone marrow-derived stromal cells (ST2 cells) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in 55-cm^2 dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. After reaching 70% confluence, the cells were detached by treatment with a 0.05% solution of trypsin, replated in 55-cm^2 dishes, 6-well (9.4 cm^2) plates, or 12-well (3.8 cm^2) plates 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, 50 units/ml penicillin, 50 μg/ml streptomycin, 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid. During the subculture, the medium was replaced every 4 days, and NOC-18 (half life, 21 h; see below for details) as the donor of NO was added at 2-day intervals as indicated in the text.

Rat calvarial osteoblast-like (ROB) cells were enzymatically isolated from the new born (day 2) Wistar rats that had been obtained from Japan SLC, Hamamatsu, Japan. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology. The constituents of the enzyme mixture, the sequential digestion procedure for the isolation of ROB cells, and the culture conditions were performed as described in a previous report (Hagiwara et al., 1996).

2.2. Detection by the reverse transcriptase-polymerase chain reaction (RT-PCR) of transcripts for various isoforms of NOS in ST2 cells

Total RNA (5 μg) was reverse transcribed by Moloney murine leukemia virus reverse transcriptase, Superscript™ (200 units; Life Technologies, Grand Island, NY, USA), using oligo(dT) primers (5 nmol) in a 20-μl reaction mixture. The cDNA was amplified in 20 μl of Taq DNA polymerase mixture (Takara, Tokyo, Japan) that contained 1 μM sense primer, 5′-AATAGAGGAACATCTGGCCAGG-3′, and antisense primer, 5′-ATGGCCGACCTGATGTTGC-3′, for mouse iNOS (258 bp) (Vandecasteele et al., 1999); 1 μM sense primer, 5′-GGCAACAGCGGCAATTTG-3′, and antisense primer, 5′-TGGACTCAGATCTAAGGCGGTTG-3′, for mouse nNOS (458 bp) (Vandecasteele et al., 1999); 1 μM sense primer, 5′-GACATTGAGAGCAAAGGGCT GC-3′, and antisense primer, 5′-CGGCTTGTCACCTCCTGG-3′, for mouse eNOS (400 bp) (Vandecasteele et al., 1999); or 1 μM sense primer, 5′-CGTTGTGGATCTGACATGCCGCC-3′, and antisense primer, 5′-CAGTGTAGCCCAGGATGCC-3′, for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control. The reaction cycle, repeated 40 times for amplification of cDNAs, consisted of incubation at 94°C for 30 s, at 65°C for 30 s, and at 72°C for 1 min. Products of PCR were subjected to electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. DNA markers (Molecular weight marker III; Boehringer Mannheim, Tokyo, Japan) were used as size markers.

2.3. Northern blot analysis

RNA was extracted from ST2 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 that contained 2.2 M formaldehyde and was then transferred to a MagnaGraph nylon membrane (Micron Separations, Westborough, MA, USA). After the membrane had been baked at 80°C for 2 h, the RNA on the membrane was allowed to hybridize overnight with cDNA for osteocalcin or GAPDH at 42°C in 50% formamide that contained 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 kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was washed twice in 1 × sodium chloride/sodium citrate (SSC) (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) that contained 0.1% SDS at room temperature for 5 min each and twice in 1 × SSC that contained 0.1% SDS at 55°C for 1 h each, then it was exposed overnight to an imaging plate. The signals on the plate were analyzed with a Bioimage Analyzer (BAS 2000; Fuji Film, Tokyo, Japan).

2.4. von Kossa staining

ST2 cells were subcultured in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and indicated reagents. The cells were fixed with 10% formaldehyde for 30 min and were 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, then treated with 5% sodium thiosulfate. Mineralized nodules were assessed with respect to their number and the total area of nodules using an automated imaging system, which consisted of a micro-

scope (IX70; Olympus, Tokyo, Japan), a camera (CCD/ICD-740; Olympus) and the Mac SCOPE program (Mitani, Fukui, Japan) (Hagiwara et al., 1996).

2.5. Quantitation of the accumulation of prostaglandin E_2 and cGMP in ST2 cells in response to NO

ST2 cells, grown in 12-well plates for 3 days (confluent cultures), were incubated at 37°C, for the indicated times, with serum-free α-MEM supplemented with 10^{-5} M NOC-18. After incubation, the amount of prostaglandin E_2 in each sample was measured with a prostaglandin E_2 enzymeimmunoassay (EIA) system (Amersham Pharmacia Biotech).

ST2 cells, grown in 12-well plates for the indicated number of days, were incubated with serum-free α-MEM supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, an inhibitor of diesterase, at 37°C for 15 min after washing with serum-free α-MEM. The cells were subsequently incubated at 37°C for 1 h with 10^{-5} M NOC-7 (see below for details) as the NO donor. Then the amount of cGMP in each sample was measured with a radioimmunoassay kit from Yamasa (Chiba, Japan).

2.6. Drugs

NOC-7 [half life, 5 min; 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine] and NOC-18 [half life, 21 h; 2,2′-(hydroxynitrosohydrazino)bis-ethanamine] were obtained from Dojindo (Kumamoto, Japan). Prostaglandin E_2, 1-(4-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid (indomethacin), and acetyl salicylic acid (aspirin) were purchased from Cayman Chemical (Ann Arbor, MI, USA). 8-Bromo-cGMP (8-Br-cGMP) and *N*-(4-nitro-2-phenoxyphenyl)-methanesulfonamide (nimesulide) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Human recombinant TNF-α was purchased from Sigma-Research Biochem. Int. (Natick, MA, USA). ^{32}P-labeled nucleotides were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). α-MEM, RPMI 1640 medium, fetal bovine serum, and penicillin/streptomycin antibiotic mixture were obtained from Life Technologies.

3. Results

3.1. Subtypes of NOS expressed in ST2 cells

We performed RT-PCR using specific primers for mouse genes for iNOS, eNOS, and nNOS in an effort to characterize the NOS/NO system in ST2 cells. As shown in Fig. 1, exposure of ST2 cells to human recombinant tumor necrosis factor-α (TNF-α; 50 ng/ml) for 24 h dramatically increased the level of mRNA for iNOS. Transcripts specific for eNOS were barely detectable by RT-PCR and we were unable to detect any transcripts specific for nNOS.

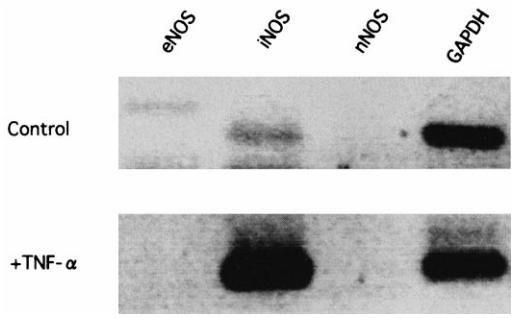

Fig. 1. Detection of a transcript specific for a single isoform of NOS in ST2 cells by RT-PCR. Total RNA was isolated from ST2 cells 3 days after subculture that had been cultured with and without TNF-α (50 ng/ml) for 24 h before harvest. Levels of mRNAs for iNOS (258 bp), eNOS (400 bp), and nNOS (458 bp) in ST2 cells were examined by RT-PCR with specific primers. After the exposure to TNF-α, there was a dramatic increase in the intensity of the signal that corresponded to iNOS mRNA. GAPDH mRNA was used as an internal control.

3.2. Effects of a donor of NO on the ascorbic acid-induced osteoblastic differentiation of ST2 cells

We reported previously that ascorbic acid induces the osteoblastic differentiation of ST2 cells (Otsuka et al., 1999). In the absence of ascorbic acid, NO generated from NOC-18 did not induce the formation of mineralized nodules, a typical characteristic of osteoblasts, by ST2 cells. By contrast, NO accelerated the formation of mineralized nodules that was induced by 50 μg/ml ascorbic acid. Therefore, in the present study, we examined the effects of NO on the osteoblastic differentiation of ST2 cells that was induced by ascorbic acid at 50 μg/ml.

Northern blot analysis revealed that continuous treatment of ST2 cells with the NO donor NOC-18 resulted in an increase in the level of mRNA for osteocalcin that was detectable on day 22 (Fig. 2). Fig. 3 shows the results of von Kossa staining of mineralized nodules formed by ST2 cells that had been treated with NOC-18, prostaglandin E_2 or 8-Br-cGMP, a membrane-permeating derivative of cGMP. At 10^{-7} M, NOC-18 increased the number and the total area of mineralized nodules by approximately 60%. The addition of prostaglandin E_2 at 10^{-9} and 10^{-7} M to the culture medium resulted in increases of 20% and 80%, respectively, in these parameters, while prostaglandin E_2 at 10^{-5} M had no effect. 8-Br-cGMP also increased in the rate of formation of mineralized nodules.

3.3. Production of prostaglandin E_2 and cGMP in response to donors of NO in ST2 cells

We examined the production of prostaglandin E_2 (Fig. 4A) and of cGMP (Fig. 4B) by ST2 cells in response to NO. In confluent ST2 cells (day 3), NOC-18 at 10^{-5} M

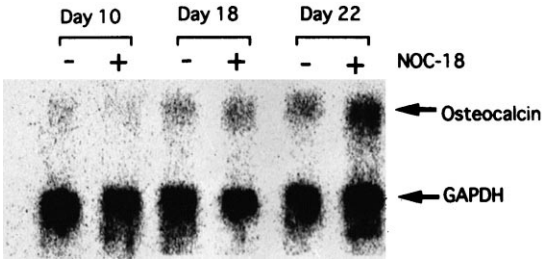

Fig. 2. Expression of mRNA for osteocalcin in ST2 cells cultured with a donor of NO. Total RNA was isolated from ST2 cells after they had been cultured in α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 10^{-5} M NOC-18 for 10, 18, and 22 days. Twenty microgram of total RNA were subjected to electrophoresis in 1% agarose gel and bands of RNA were allowed to hybridize with ^{32}P-labeled cDNA for osteocalcin as described in the text. Expression of the mRNA for GAPDH was monitored as an internal reference. Data are representative of results from three different experiments.

enhanced the rate of production of prostaglandin E_2 in a time-dependent manner over the course of 24 h (Fig. 4A).

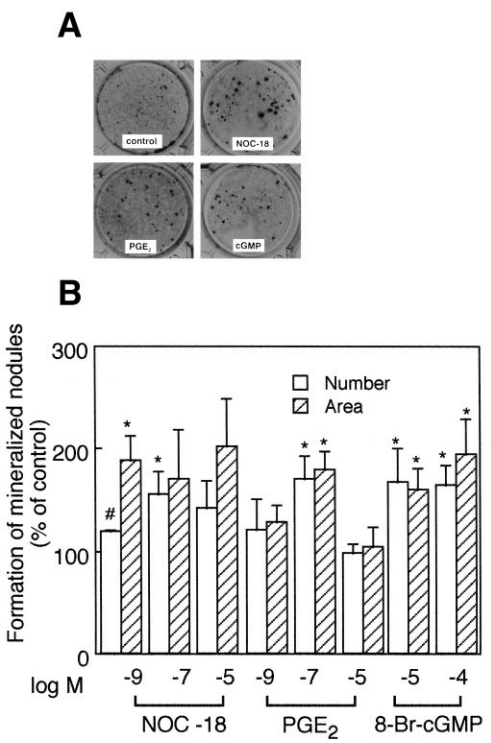

Fig. 3. (A) Phase-contrast photomicrographs of mineralized nodules in cultures of ST2 cells that had been treated with NOC-18, prostaglandin E_2 and 8-Br-cGMP, respectively. ST2 cells in 6-well plates were cultured for 22 days with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid and the compound to be tested. Mineralized nodules were subjected to von Kossa staining as described in the text. Arrows indicate stained mineralized nodules. (B) Results of quantitative analysis. Numbers and areas of mineralized nodules were determined with the Mac SCOPE program, as described in the text. Data are means ± S.E. of results from the three wells and are representative of results from four different experiments. * $P < 0.05$ vs. control; # $P < 0.01$ vs. control.

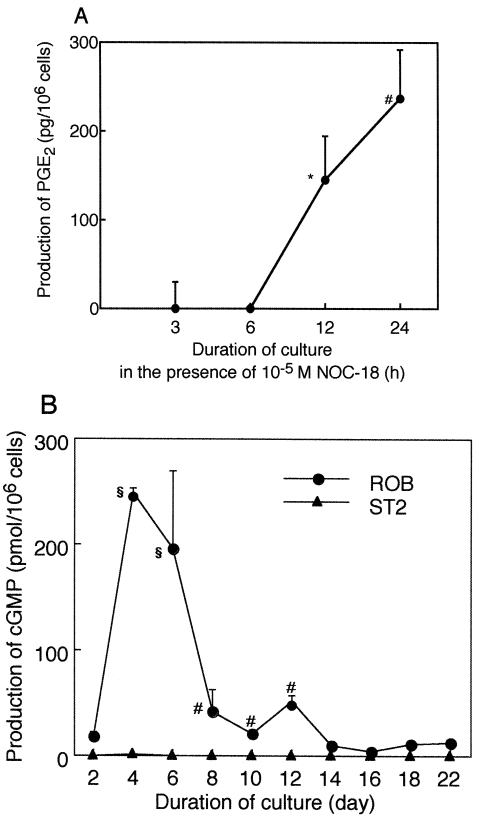

Fig. 4. (A) Production of prostaglandin E_2 in response to an NO donor in ST2 cells. ST2 cells in 12-well plates were cultured for 3 days with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. Cells were exposed to 10^{-5} M NOC-18 (half life, 21 h) for the indicated times and levels of prostaglandin E_2 were defined as the difference between the production of prostaglandin E_2 in the presence and the absence of NOC-18. Each value is the mean ± S.E. of the results from the three different wells and is representative of results from the three different experiments. * $P < 0.05$ vs. results obtained at 0 h; # $P < 0.01$ vs. results obtained at 0 h. (B) Production of cGMP. ST2 cells and ROB cells in 12-well plates were cultured for the indicated times with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. Cells were exposed to 10^{-5} M NOC-7 (half life, 5 min) for 1 h in the presence of 0.5 mM 3-isobutyl-1-methylxanthine and then levels of cGMP were determined as described in the text. Each value is the mean ± S.E. of results from the three different wells and is representative of the results from the three different experiments. # $P < 0.01$ vs. results obtained at day 2; § $P < 0.001$ vs. results obtained at day 2.

However, the production of prostaglandin E_2 in response to NO was no longer detectable after 6 days of culture (data not shown). In cGMP assay, we obtained the results that 10^{-5} M NOC-18 has no effects on the production of cGMP by ST2 cells. We considered that NOC-18 is not a suitable NO donor because the incubation time for cGMP production was 1 h. Therefore, we used NOC-7 (half life of 5 min) instead of NOC-18 (half life of 21 h). However, NOC-7 at 10^{-5} M also had no effects on cGMP production by soluble guanylate cyclase in ST2 cells during the 22 days of the experiments (Fig. 4B). By contrast, in ROB cells, the production of cGMP in response to NOC-7 clearly peaked at the early stage (days 4–6) of culture.

3.4. Effects of cyclooxygenase inhibitors on the formation of mineralized nodules by ST2 cells

To confirm the participation in prostaglandins on the formation of mineralized nodules by ST2 cells, we examined the effects of cyclooxygenase inhibitors, namely, aspirin (Mitchell et al., 1994) (a specific inhibitor of cyclooxygenase-1), indomethacin (a specific inhibitor of both cyclooxygenase-1 and cyclooxygenase-2), and nimesulide (Sawdy et al., 1998)(a specific inhibitor of cyclooxygenase-2). Continuous exposure to indomethacin and to nimesulide at 10^{-5} M inhibited the basal and NO-induced formation of mineralized nodules by ST2 cells, while aspirin at 10^{-5} M had no effect on this process (Fig. 5A). Fig. 5B shows that nimesulide inhibited the formation of mineralized nodules in a dose-dependent manner.

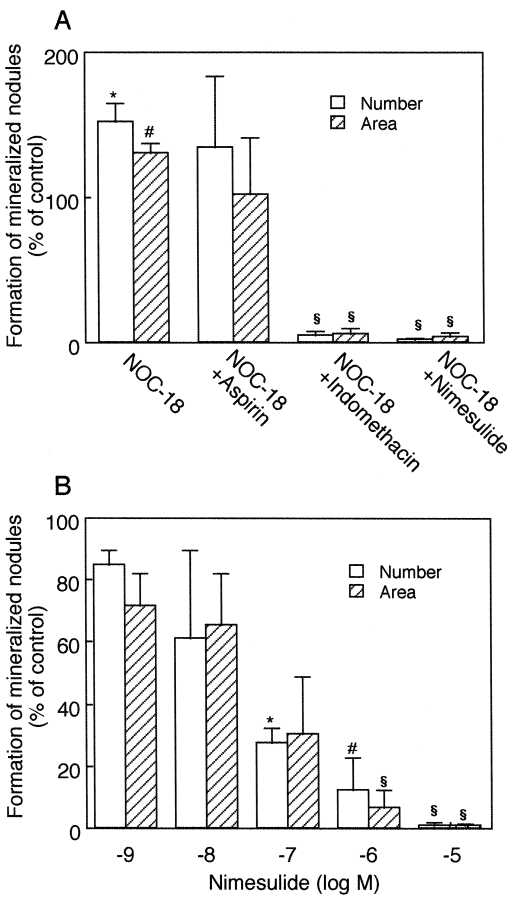

Fig. 5. Effects of inhibitors for cyclooxygenases on the formation of mineralized nodules by ST2 cells. ST2 cells in 6-well plates were cultured for 23 days with α-MEM that contained 10% fetal bovine serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and with and without 10^{-5} M NOC-18. (A) The effects of each cyclooxygenase inhibitor at 10^{-5} M on the formation of mineralized nodules by ST2 cells treated with NOC-18. * $P < 0.05$ vs. control; # $P < 0.01$ vs. control; § $P < 0.001$ vs. results obtained with NOC-18. (B) The dose-dependency of the effect of nimesulide on ST2 cells. Each value is the mean ± S.E. of results from the three different wells and is representative of the results from the two different experiments. * $P < 0.05$ vs. control; # $P < 0.01$ vs. control; § $P < 0.001$ vs. control.

4. Discussion

The formation within cells of NO by NOS and the subsequent stimulation of cyclooxygenases and soluble guanylate cyclase are important pathways in the regulation of cell function and cell communication. However, information about the subtypes of NOS in stromal ST2 cells and the effects of NO on ST2 cells has been limited. In the present study, we identified the mRNA for inducible-type NOS in ST2 cells by RT-PCR. Our results suggest that NO produced in response to stimulation by cytokines might act on ST2 cells in an autocrine fashion. Moreover, we found that NO accelerated the osteoblastic differentiation of ST2 cells that occurs in response to ascorbic acid. It has been reported that NO regulates the differentiation of and/or the deposition of Ca^{2+} in cells committed to the formation of osteoblasts, such as rat (Otsuka et al., 1998) and mouse (Chae et al., 1997; Hikiji et al., 1997) calvarial osteoblast-like cells, mouse clonal preosteoblasts (MC3T3-E1 cells) (Kanematsu et al., 1997) and a line of rat osteosarcoma cells (ROS 17/2.8) (Chae et al., 1997). The role of NO in the differentiation of osteoblastic cells, other than the regulation of the production of prostaglandin E_2 and/or cGMP, remains unclear.

In ST2 cells, we failed to detect an increase in the activity of soluble guanylate cyclase in response to NO but NO induced the synthesis of prostaglandin E_2 in a time-dependent manner. Prostaglandin E_2 has been reported to have a stimulatory (Kumegawa et al., 1984; Fujieda et al., 1999; Kaneki et al., 1999) or a biphasic (Hakeda et al., 1985a, 1987; Raisz and Fall, 1990) effect on osteoblastic differentiation through the inositol phosphates/Ca^{2+} pathway and/or cAMP-dependent mechanism. Receptors for prostaglandin E_2 can be divided into four subtypes, EP_1–EP_4 (Coleman et al., 1994), and signaling by prostaglandin E_2 differs according to the receptor that is expressed on cells (Suda et al., 1996). In the present study, we showed that NO stimulated the production of prostaglandin E_2 by ST2 cells and prostaglandin E_2 might act on ST2 cells in an autocrine/paracrine manner. The autocrine actions of prostaglandins are well known in osteoblast-like cells; prostaglandin E_2 that is produced by the stimulation of interleukin 1 and TNF-α (Kanematsu et al., 1997) and 1,25-$(OH)_2$ vitamin D_3 (Schwartz et al., 1992) regulates the differentiation of MC3T3-E1 cells in an autocrine fashion. We found that exogenous prostaglandin E_2 at 10^{-9} M increased the formation of mineralized nodules of ST2 cells by 20%, as shown in Fig. 3. The concentration of prostaglandin E_2 in the conditioned medium of ST2 cells that had been treated with an NO donor for 24 h reached approximately 0.7×10^{-9} M (Fig. 4), which might be sufficient for local acceleration of the formation of mineralized nodules by ST2 cells. We also showed that a short-term release of prostaglandin E_2 by ST2 cells could affect the formation of mineralized nodules. Similar effects of prostaglandin E_2 was observed in rat calvarial os-

teoblasts (Fujieda et al., 1999). Moreover, a short-term response of mouse preosteoblastic MC3T3-E1 cells to endothelin-1 (Hiruma et al., 1998) and C-type natriuretic peptide (Inoue et al.,1999) have been reported to regulate long-term functional responses, such as the activity of alkaline phosphatase and the formation of mineralized nodules. However, the mechanism of action by those factors remains to be elucidated. We are now examining to identify, by the differential display-PCR method, the genes whose expression is regulated by those factors.

We confirmed the stimulatory effects of prostaglandin E_2 on the formation of mineralized nodules by ST2 cells using specific inhibitors for cyclooxygenases. Two inhibitors of cyclooxygenase-2, indomethacin and nimesulide, inhibited the basal and NO-induced formation of mineralized nodules by ST2 cells, while aspirin, an inhibitor of cyclooxygenase-1, did not inhibit this process (Fig. 5). Our results suggest that a signaling pathway for the production of prostaglandins might be involved in the ascorbic acid-induced formation of mineralized nodules by ST2 cells, both at the basal level and at the elevated level observed in response to NO. We proposed previously that ascorbic acid might induce the osteoblastic differentiation of ST2 cells through the formation of the collagen matrix and the action of bone morphogenetic proteins (Otsuka et al., 1999). Prostaglandin E_2 stimulates the synthesis of the collagen matrix by preosteoblastic MC3T3-E1 cells (Hakeda et al., 1985b) and by organ cultures of fetal rat calvariae (Raisz and Fall, 1990). It might be possible that the synthesis of the collagen matrix in response to prostaglandins contributes to the formation of mineralized nodules by stromal cells. The relationships between the roles of bone morphogenetic proteins and prostaglandin E_2 remain to be clarified. The proliferation of MC3T3-E1 cells is mediated via the prostaglandin E_2 (Hakeda et al., 1987). Prostaglandin E_2 was also reported to regulate, via cAMP, the secretion of macrophage colony stimulating factor by human bone marrow stromal cells (Besse et al., 1999). Potential effects via proliferation and the production of matrix protein by prostaglandin E_2 on the osteoblastic differentiation of ST2 cells remain to be characterized in detail.

There are two primary pathways for the production of cGMP: one involves the activation of soluble guanylate cyclase by NO; and the other involves activation of membrane-bound guanylate cyclases, such as the A-type natriuretic peptide receptor and the B-type natriuretic peptide receptor, by natriuretic peptides (Hagiwara et al., 1995). Both the 8-Br-cGMP (Inoue et al., 1995; Hagiwara et al., 1996) and natriuretic peptides (Hagiwara et al., 1996) promote the differentiation and mineralization of osteoblastic cells. In the present study, the addition of 8-Br-cGMP to the culture medium of ST2 cells enhanced the formation of mineralized nodules. However, cGMP was not produced in response to NO by ST2 cells. Thus, cGMP potentiates the osteoblastic differentiation of ST2 cells, but the action of NO in ST2 cells is independent of the generation of cGMP.

In conclusion, NO accelerated the osteoblastic differentiation of stromal ST2 cells and it appears that the NO-stimulated differentiation of ST2 cells might be due to the production of prostaglandin E_2. NO might be involved in the pathogenesis of bone loss in diseases associated with the activation of cytokines, such as periodontal disease and rheumatoid arthritis. Our findings suggest that NO might participate in bone metabolism by promoting osteoblastic differentiation.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan and by grants from the ''Ground Research Announcement for the Space Utilization'' promoted by NASDA and the Japan Space Forum.

References

Besse, A., Trimoreau, F., Faucher, J.L., Praloran, V., Denizot, Y., 1999. Prostaglandin E_2 regulates macrophage colony stimulating factor secretion by human bone marrow stromal cells. Biochim. Biophys. Acta 1450, 444–451.

Chae, H.-J., Park, R.-K., Chung, H.-T., Kang, J.-S., Kim, M.-S., Choi, D.-Y., Bang, B.-G., Kim, H.-R., 1997. Nitric oxide is a regulator of bone remodeling. J. Pharm. Pharmacol. 49, 897–902.

Chen, D., Harris, X., Ji, M.A., Feng, J.Q., Karsenty, G., Celeste, A.J., Rosen, V., Mundy, G.R., Harris, S.E., 1998. Differential roles for bone morphogenetic protein (BMP) receptor type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages. J. Cell Biol. 142, 295–305.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Coleman, R.A., Smith, W.L., Narumiya, S., 1994. Classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol. Rev. 46, 205–229.

Damoulis, P.D., Hauschka, P.V., 1994. Cytokines induce nitric oxide production in mouse osteoblasts. Biochem. Biophys. Res. Commun. 201, 924–931.

Fujieda, M., Kiriu, M., Mizuochi, S., Hagiya, K., Kaneki, H., Ide, H., 1999. Formation of mineralized bone nodules by rat calvarial osteoblasts decreases with donor age due to a reduction in signaling through EP_1 subtype of prostaglandin E_2 receptor. J. Cell. Biochem. 75, 215–225.

Hagiwara, H., Hirose, S., Takei, Y., 1995. Natriuretic peptides and their receptors. Zool. Sci. 12, 141–149.

Hagiwara, H., Inoue, A., Yamaguchi, A., Yokose, S., Furuya, M., Tanaka, S., Hirose, S., 1996. cGMP produced in response to ANP and CNP regulates proliferation and differentiation of osteoblastic cells. Am. J. Physiol. 270, C1311–C1318.

Hakeda, Y., Hotta, T., Kurihara, N., Ikeda, E., Maeda, N., Yagyu, Y., Kumegawa, M., 1987. Prostaglandin E_1 and $F_{2\alpha}$ stimulate differentiation and proliferation, respectively, of clonal osteoblastic MC3T3-E1 cell by different second messengers in vitro. Endocrinology 121, 1966–1974.

Hakeda, Y., Nakatani, Y., Hiramatsu, M., Kurihara, N., Tsunoi, M., Ikeda, E., Kumegawa, M., 1985a. Inductive effects of prostaglandins on alkaline phosphatase in osteoblastic cells, clone MC3T3-E1. J. Biochem. 97, 97–104.

Hakeda, Y., Nakatani, Y., Kurihara, N., Ikeda, E., Maeda, N., Kumegawa, M., 1985b. Prostaglandin E_2 stimulates collagen and non-collagen protein synthesis and prolyl hydroxylase activity in osteoblastic clone MC3T3-E1 cells. Biochem. Biophys. Res. Commun. 126, 340–345.

Helfrich, M.H., Evans, D.E., Grabowski, P.S., Pollock, J.S., Ohshima, H., Ralston, S.H., 1997. Expression of nitric oxide synthase isoforms in bone and bone cell cultures. J. Bone Miner. Res. 12, 1108–1115.

Hikiji, H., Shin, W.S., Oida, S., Takato, T., Koizumi, T., Toyo-oka, T., 1997. Direct action of nitric oxide on osteoblastic differentiation. FEBS Lett. 410, 238–242.

Hiruma, Y., Inoue, A., Shiohama, A., Otsuka, E., Hirose, S., Yamaguchi, A., Hagiwara, H., 1998. Endothelins inhibit the mineralization of osteoblastic MC3T3-E1 cells through the A-type endothelin receptor. Am. J. Physiol. 275, R1099–R1105.

Inoue, A., Hayakawa, T., Otsuka, E., Kamiya, A., Suzuki, Y., Hirose, S., Hagiwara, H., 1999. Correlation between induction of expression of biglycan and mineralization by C-type natriuretic peptide in osteoblastic cells. J. Biochem. 125, 103–108.

Inoue, A., Hiruma, Y., Hirose, S., Yamaguchi, A., Hagiwara, H., 1995. Reciprocal regulation by cyclic nucleotides of the differentiation of rat osteoblast-like cells and mineralization of nodules. Biochem. Biophys. Res. Commun. 215, 1104–1110.

Kaneki, H., Takasugi, I., Fujieda, M., Kiriu, M., Mizuochi, S., Ide, H., 1999. Prostaglandin E_2 stimulates the formation of mineralized bone nodules by a cAMP-independent mechanism in the culture of adult rat calvarial osteoblasts. J. Cell. Biochem. 73, 36–48.

Kanematsu, M., Ikeda, K., Yamada, Y., 1997. Interaction between nitric oxide synthase and cyclooxygenase pathways in osteoblastic MC3T3-E1 cells. J. Bone Miner. Res. 12, 1789–1796.

Kumegawa, M., Ikeda, E., Tanaka, S., Haneji, T., Yora, T., Sakagishi, Y., Minami, N., Hiramatsu, M., 1984. The effects of prostaglandin E_2, parathyroid hormone, 1,25 dihydroxycholecalciferol, and cyclic nucleotide analogs on alkaline phosphatase activity in osteoblastic cells. Calcif. Tissue Int. 36, 72–76.

Lowik, C.W.G.M., Nibbering, P.H., van deRuit, M., Papapoulos, S.E., 1994. Inducible production of nitric oxide in osteoblast-like cells and in fetal mouse bone explants is associated with suppression of osteoclastic bone resorption. J. Clin. Invest. 93, 1465–1472.

Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1994. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenases. Proc. Natl. Acad. Sci. U.S.A. 90, 11693–11697.

Moncada, S., Palmer, R.M.J., Hibbs, J.R., Higgs, A.E., 1991. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 43, 109–142.

Natsume, T., Tomita, S., Iemura, S., Kinto, N., Yamaguchi, A., Ueno, N., 1997. Interaction between soluble type I receptor for bone morphogenetic protein and bone morphogenetic protein-4. J. Biol. Chem. 272, 11535–11540.

Ogawa, M., Nishikawa, S., Ikuta, K., Yamamura, F., Naito, M., Takahashi, K., 1988. B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. EMBO J. 7, 1337–1343.

Otsuka, E., Hirano, K., Matsushita, S., Inoue, A., Hirose, S., Yamaguchi, A., Hagiwara, H., 1998. Effects of nitric oxide from exogenous nitric oxide donors on osteoblastic metabolism. Eur. J. Pharmacol. 349, 345–350.

Otsuka, E., Yamaguchi, A., Hirose, S., Hagiwara, H., 1999. Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. Am. J. Physiol. 277, C132–C138.

Raisz, L.G., Fall, P.M., 1990. Biphasic effects of prostaglandin E_2 on bone formation in cultured fetal rat calvariae: interaction with cortisol. Endocrinology 126, 1654–1659.

Ralston, S.H., Grabowski, P.S., 1996. Mechanisms of cytokine-induced bone resorption: roles of nitric oxide, cyclic guanosine monophosphate, and prostaglandins. Bone 19, 29–33.

Sato, T., Morita, I., Murota, S., 1998. Involvement of cholesterol in osteoclast-like cell formation via cellular fusion. Bone 23, 135–140.

Sawdy, R., Knock, G.A., Bennett, P.R., Poston, L., Aaronson, P.I., 1998. Effect of nimesulide and indomethacin on contractility and the Ca^{2+} channel current in myometrial smooth muscle from pregnant women. Br. J. Pharmacol. 125, 1212–1217.

Schwartz, Z., Dennis, R., Bonewald, L., Swain, L., Gomez, R., Boyan, B.D., 1992. Differential regulation of prostaglandin E_2 synthesis and phospholipase A_2 activity by 1,25-$(OH)_2D_3$ in three osteoblast-like cell lines (MC3T3-E1, ROS 17/2.8, and MG-63). Bone 13, 51–58.

Suda, M., Tanaka, K., Natsui, K., Usui, T., Tanaka, I., Fukushima, M., Shigeno, C., Konishi, J., Narumiya, S., Ichikawa, A., Nakao, K., 1996. Prostaglandin E receptor subtypes in mouse osteoblastic cell line. Endocrinology 137, 1698–1705.

Vandecasteele, G., Eschenhagen, T., Scholz, H., Stein, B., Verde, I., Fischmeister, R., 1999. Muscarinic and β-adrenergic regulation of heart rate, force of contraction and calcium current is preserved in mice lacking endothelial nitric oxide synthase. Nat. Med. 5, 331–334.

Yamaguchi, A., Ishizuya, T., Kintou, N., Wada, T., Katagiri, T., Wozney, J.M., Rosen, V., Yoshiki, S., 1996. Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. Biochem. Biophys. Res. Commun. 220, 366–371.