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Direct action of nitric oxide on osteoblastic differentiation

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Abstract The effect of nitric oxide (NO) on osteoblastic differentiation was examined in cultured mouse osteoblasts. Interleukin-1β and tumor necrosis factor-α expressed inducible NO synthase gene with little effect on constitutive NO synthase gene. These cytokines increased NO production, which was inhibited by L-NMMA pretreatment, and decreased alkaline phosphatase (AlPase) activity, which was not restored by L-NMMA. Furthermore, NO donors, sodium nitroprusside and NONOate dose-dependently elevated AlPase activity and expression of osteocalcin gene. These results suggest that NO directly facilitates osteoblastic differentiation and the cytokine-induced inhibition of AlPase activity is mediated via mechanism other than NO.

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Key words: NO; NOS gene expression; Osteoblastic differentiation; TNF-α; IL-1β

1. Introduction

Nitric oxide (NO) is involved in various pathophysiological processes in many tissues [1] and is produced from L-arginine by nitric oxide synthase (NOS). So far, three isoforms of NOS are isolated. Two constitutive isozymes; endothelial cell (ecNOS) and neuronal (nNOS) types which produce less amount of NO with several physical/chemical stimuli, while inducible isoform (iNOS) yields larger amount of NO through de novo synthesis of the enzyme in response to proinflammatory cytokines or bacterial endotoxin [1].

Osteoblasts have been reported to produce NO after induction of iNOS gene by cytokines [2,3], and NO may inhibit the bone-resorbing activity in adjacent osteoclasts [2,4], suggesting a cross-talk between osteoblast and osteoclast via NO. Nevertheless, the role of NO in osteoblasts is still obscure. The purpose of the present study was addressed to examine whether (1) mouse osteoblast expresses constitutive (endothelial cell type) and/or inducible NOS gene, (2) these genes actually increase NO production and (3) NO actively affects the osteoblastic differentiation, with using four parameters, alkaline phosphatase (AlPase) activity and cGMP levels in osteoblasts, an expression of osteocalcin gene and PGE_2 synthesis in culture medium.

2. Materials and methods

2.1. Cell culture

Primary cultures of mouse osteoblasts were prepared from 1-day-old ddy mouse calvaria as described previously [5]. Isolated cells were grown in α MEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Bioserum, Victoria, Australia), penicillin, streptomycin and amphotericin B (Sigma, St. Louis, MO, USA). When indicated, N^G-monomethyl-L-arginine (L-NMMA, Wako, Osaka, Japan, 10^{-4} M) was applied to the culture medium 4 days before and throughout cytokine stimulation described below.

2.2. Reverse transcription-polymerase chain reaction

Each NOS message was detected using RT-PCR as described previously [6]. Total RNA was extracted, and the reverse transcribed cDNA was used as a template for PCR. The primer sequences for ecNOS were: upper, 5′-GGCATCACCAGGAAGAAGAC (1516–1535); and lower, 5′-ACTGGACTCCTTCCTCTTCC (1953–1934) [6]. The primer sequences for iNOS were: upper, 5′-GGAAAAGGACATTAACAACAA (244–264); and lower, 5′-ATGTACCAGCCATTGAAGGGG (1287–1267) [7].

Diethylamine NONOate (Cayman Chemical, Ann Arbor, MI, USA) was applied to osteoblasts for 48 h and the total RNA was extracted and used for detection of osteocalcin message. The primer sequences for osteocalcin were: upper, 5′-ATGAGGACCCTCTCTCTGCT (49–69); and lower, 5′-CCGTAGATGCGTTTGTAGGC (325–305) [8].

The annealing/elongating/denaturating conditions for the PCR reaction was 56/72/94°C for a total of 25 cycles, 55/72/94°C for a total of 30 cycles, 55/72/94°C for a total of 35 cycles with an initial 5 min denaturation and an additional 10 min (7 min for osteocalcin) extension step at 72°C for ecNOS, iNOS and osteocalcin, respectively. The reaction products were separated by gel electrophoresis and stained in ethidium bromide.

2.3. Assays of nitrate/nitrite, cGMP, AlPase and prostaglandin E_2 (PGE_2)

NO was measured as nitrate/nitrite products in medium 48 h after the incubation with or without recombinant tumor necrosis factor-α (TNF-α, 100 ng/ml, Dainippon Pharmaceutical, Tokyo, Japan) and/or interleukin-1β (IL-1β, 10 ng/ml, Genzyme, Cambridge, MA, USA). Nitrate was converted to nitrite with nitrate reductase, then Griess reagent was applied for spectrophotometric measurement at 540 nm [9]. Nitrite level was normalized with protein amount measured by Bradford's method (Bio-Rad, Germany).

NO action was verified with measurement of cGMP with or without addition of an NO donor, sodium nitroprusside (SNP, Wako). For cGMP assay, confluent cells were preincubated at 37°C for 20 min then with 0.1 mM 3-isobutyl-1-methylxanthine (Wako) for 6 min. After 2 min of administration of SNP, incubation was terminated with ice-cold 10% trichloroacetic acid. cGMP levels were determined using a EIA kit (Cayman Chemical).

In bone tissues, the expression of AlPase is closely associated with osteoblastic differentiation [10]. Osteoblasts applied SNP for 5 h or cytokines for 48 h were washed twice with phosphate-buffered saline and then lysed in 0.1% Triton X-100. An aliquot of homogenate after three cycles of freezing and thawing was assayed for AlPase activity

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Abbreviations: AlPase, alkaline phosphatase; cGMP, cyclic guanosine monophosphate; diethylamine NONOate, Ethanamine, *N*-ethyl-, compound with 1,1-diethyl-2-hydroxy-2-nitrosohydrazine; EIA, enzyme immunoassay; IL-1β, Interleukin-1β; L-NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; RT-PCR, reverse transcription-polymerase chain reaction; SNP, sodium nitroprusside; TNF-α, tumor necrosis factor-α

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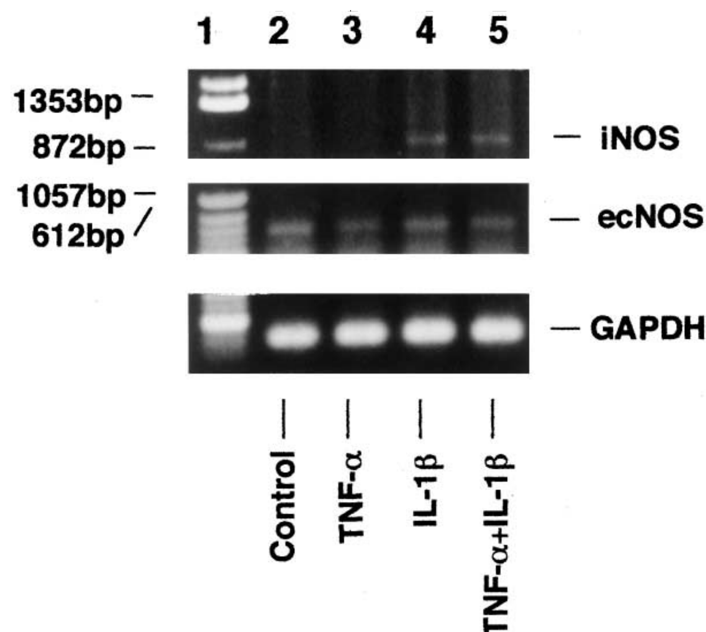

Fig. 1. Expression of iNOS, ecNOS and GAPDH mRNA in unstimulated cells or cells stimulated with several factors. Isolated mRNA was reverse transcribed and amplified by PCR. The PCR product showed the 526-bp and 1044-bp for ecNOS for iNOS of predicted size, respectively. Lanes: 1, DNA size markers; 2, unstimulated osteoblasts; 3, TNF-α-treated cells; 4, IL-1β-treated cells; 5, TNF-α plus IL-1β-treated cells.

(Wako, Osaka). The content of PGE_2 in culture media of the osteoblasts were measured using a EIA kit (Cayman Chemical).

2.4. Statistics

All values were expressed as the mean ± SE. Statistical differences between the values were examined by one-way ANOVA for multiple comparisons followed by Fisher's test. The p values less than 0.05 were considered significant.

3. Results

3.1. Induction of mRNA of iNOS and ecNOS by cytokines

We used RT-PCR to define the isoforms of NOS specifically expressed in mouse osteoblasts (Fig. 1). iNOS mRNA was not detected in unstimulated cells or cells treated with TNF-α (100 ng/ml) alone. In contrast, IL-1β (10 ng/ml) and combinations of two cytokines (TNF-α+IL-β) induced the iNOS mRNA expression (a 1044-bp PCR product in Fig. 1). In contrast, ecNOS mRNA was detected in both unstimulated and stimulated osteoblasts (a 526-bp PCR product in Fig. 1). These data suggest that iNOS gene was exclusively induced by IL-1β, but not by TNF-α alone, while ecNOS gene was constitutively expressed, irrespective of the stimulation with these cytokines.

3.2. Modulation of NO production and AIPase activity with cytokines

Without cytokine, mouse osteoblasts released a modest but steady amount of NO detected as nitrate/nitrite (25.7 ± 5.5 nmol/mg protein, Fig. 2). TNF-α (100 ng/ml) alone had no effect on the basal release (25.0 ± 5.0 nmol/mg protein). In contrast, IL-1β (10 ng/ml) increased the NO production to 46.6 ± 5.3 nmol/mg protein ($p < 0.02$, vs. control). Combination of TNF-α and IL-1β enhanced the NO production three folds over the control level (70.7 ± 7.2 nmol/mg protein, $p < 0.0001$, vs. control), indicating that IL-1β synergistically increased NO production with TNF-α via NOS induction. The increased NO production by combination of TNF-α plus IL-1β was attenuated by a competitive NOS inhibitor, L-NMMA (10^{-4} M, Fig. 3A), and decreased from 75.9 ± 2.6 nmol/mg protein to 36.1 ± 2.6 nmol/mg protein ($p < 0.05$, vs. TNF-α+IL-1β).

Combination of TNF-α and IL-1β reduced AIPase activity in osteoblasts (77.4 ± 4.6 vs. 56.6 ± 2.7 nmol/min per mg protein). These results are compatible with the previous reports that TNF-α and/or IL-1β possess bone-resorbing action [11,12]. However, L-NMMA did not restore the reduced level of AIPase by these cytokines at all (54.9 ± 2.6 nmol/min per mg protein, Fig. 3B). These results indicate that the bone-resorbing effect of cytokines is not mediated via NO, despite of cytokine induction of iNOS gene and the actual NO production.

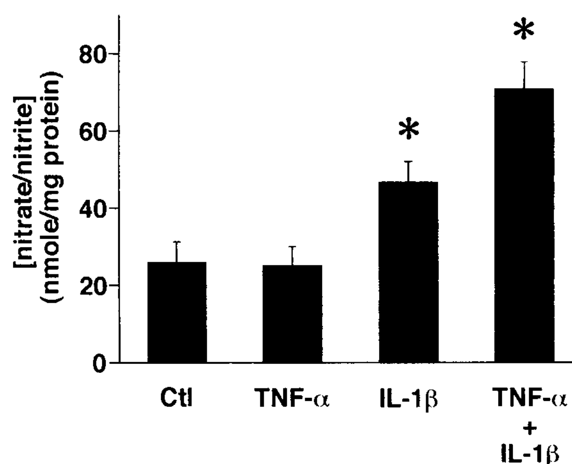

Fig. 2. Stimulatory effects of TNF-α and IL-1β on NO production, measured as nitrate/nitrite in osteoblasts (mean ± SE, $n = 12$). * indicates a significant difference ($p < 0.02$), compared with the control (Ctl).

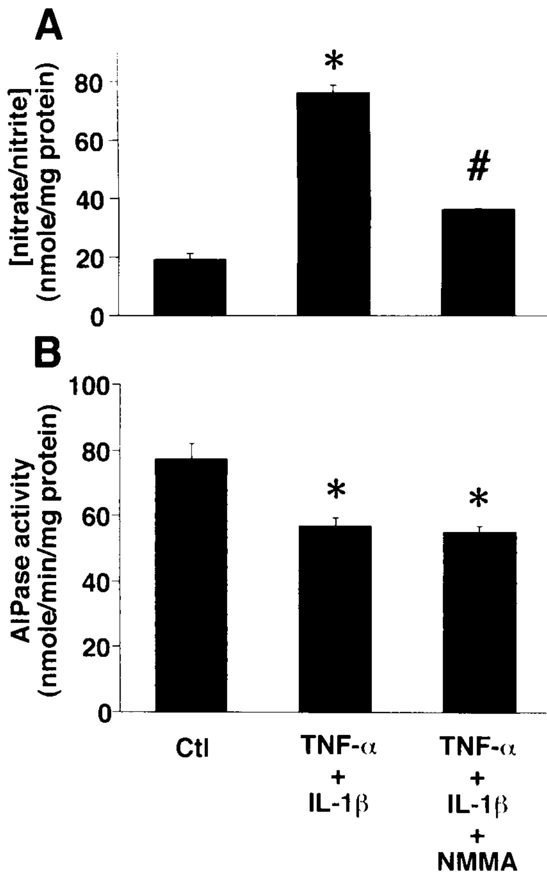

Fig. 3. Inhibitory effect of L-NMMA on NO production (A) and alkaline phosphatase (AlP) activity in culture medium (B). * and # indicate a significant difference ($p < 0.001$), compared with the control and condition stimulated by TNF-α plus IL-1β, respectively. Each value denotes the mean ± SE ($n = 6$).

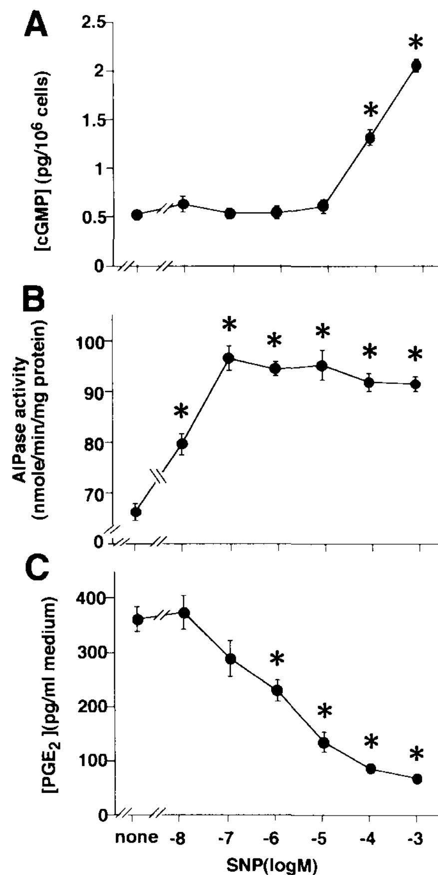

Fig. 4. Effects of SNP on cGMP levels (mean ± SE, $n = 5$) (A), alkaline phosphatase (AlP) activity (mean ± SE, $n = 10$) (B), and PGE_2 production (mean ± SE, $n = 5$) (C) in osteoblasts. * denotes a significant difference ($p < 0.001$), compared with the control.

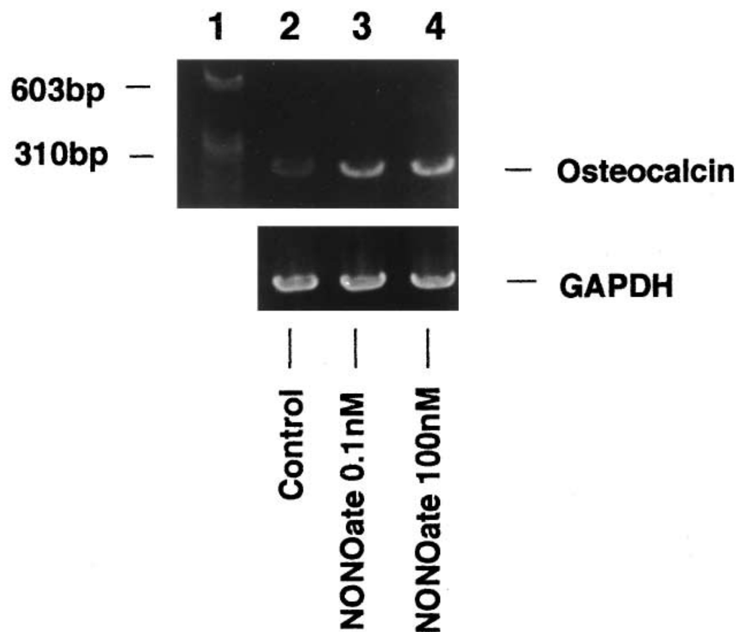

Fig. 5. Expression of osteocalcin and GAPDh mRNA in unstimulated cells or cells stimulated with diethylamine NONOate. Isolated mRNA was reverse transcribed and amplified by PCR. The PCR product was 276 bp. Lanes: 1, DNA size markers; 2, unstimulated osteoblasts; 3, 0.1 nM diethylamine NONOate-treated cells; 4, 100 nM diethylamine NONOate-treated cells.

3.3. Effect of NO donors on biosynthesis of cGMP, AlPase activity and PGE_2 production, and osteocalcin gene expression

To examine the direct effect of NO on osteoblasts, we used the NO donor, SNP or diethylamine NONOate [13]. Two-minute incubation of SNP ($>10^{-4}$ M) increased intracellular cGMP level in concentration-dependent manner (Fig. 4A). This result confirms our previous report that NO has an autocrine action on the NO producing cells themselves, as well as adjacent cells via paracrine action [14,15].

Osteoblasts treated with SNP for 5 h, did elevate AlPase activity in a concentration-dependent manner (Fig. 4B), exhibiting a clear contrast with the reduction of the activity in cytokine-stimulated osteoblasts (Fig. 3B). In response to SNP, the level of PGE_2 in culture medium decreased in a concentration-dependent manner (Fig. 4C). The SNP concentrations necessary for 50% increment for cGMP synthesis and AlPase activity were 10^{-4} M and 10^{-8} M, respectively.

The expression of osteocalcin mRNA was enhanced in osteoblasts when they were treated by a long-lasting NO donor, diethylamine NONOate, for 48 h more than untreated osteoblasts (a 276-bp PCR product in Fig. 5).

4. Discussion

Rat osteosarcoma cells have been reported to express nNOS [16]. However, the expression of ecNOS in osteoblasts have not been reported thus far. We have found that mouse osteoblasts express ecNOS gene constitutively and iNOS gene only after cytokine stimulation. We have also found ecNOS and iNOS in human osteosarcoma cells (data not shown). Furthermore, homogeneous staining was observed in mouse-osteoblast culture with specific antibody against ecNOS (data not shown). These results show that ecNOS expresses in mouse osteoblasts at both mRNA and protein level. The cytokine-induced NO production was inhibited by L-NMMA. These cytokines also decreased osteoblastic differentiation in terms of reduction of AlPase activity without restoration by L-NMMA. Present results were totally different from the previous report by Hukkanen et al. [3] in the aspect of L-NMMA action on cytokine-induced AlPase activity and accordingly give rise to an argument against the scheme that NO facilitates osteoblastic differentiation.

The role of steady ecNOS gene expression and low but significant concentrations of NO produced in unstimulated cells (Fig. 2) has not been noticed so far. Low concentrations of NO produced by ecNOS may control the basal metabolism of osteoblasts. In addition to the basal synthesis of NO by ecNOS, large amount of NO may have been produced by iNOS after the induction by IL-1β and TNF-α+IL-1β (Figs. 2 and 3A). This situation will take place in the setting of inflammatory response of osteoblastic metabolism.

iNOS is not an only gene induced by IL-1β and/or TNF-α in osteoblasts. IL-1β induces cyclic AMP production in human osteoblasts [17]. TNF-α also promotes the expression of prostaglandin endoperoxide synthase-2 mRNA and synthesis of PGE_2 [18]. In other tissues, such as vascular tissues, the condition which evokes NO simultaneously produces superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$) [19]. There is no evidence that the cytokine-induced NO has direct action on bone resorption.

Accordingly, it is of a great significance to know the direct effect of NO on osteoblasts. An NO donor, SNP, increased the AlPase activity, which is the index of osteoblastic differentiation, in a concentration-dependent manner. It also increased the intracellular concentration of cGMP, which is the second messenger of NO as well as the activator of the osteoblastic differentiation [20,21]. The long-lasting NO donor, diethylamine NONOate, increased the gene expression of osteocalcin, which is the another index of osteoblastic differentiation [22,23]. On the contrary, it decreased the levels of PGE_2 in medium. Prostaglandins are multifunctional regulators with stimulatory and inhibitory effects on bone metabolism [24]. At low concentrations, PGE_2 increased collagen synthesis in cultured fetal rat calvaria, whereas at high concentrations, the major effect was inhibitory. However, as to the osteoblastic differentiation, PGE_2 is reported to be inhibitory [25]. Present findings indicate that NO acts on osteoblastic differentiation and suggest that NO inhibits bone resorption through PGE_2 production by osteoblasts.

SNP below 10^{-4} M changed the levels of both AlPase activity and PGE_2 in medium, while the same agent above 10^{-4} M raised the intracellular concentration of cGMP, the second messenger of NO. These results suggest that the effect of NO on osteoblastic differentiation is mediated by both cGMP-dependent and independent mechanisms. Actually, NO action is not simply cGMP-dependent, and cGMP-independent pathway also plays significant role on platelets, fibroblasts and osteoclasts [4,26,27].

In conclusion, we have demonstrated that NO directly facilitates osteoblastic differentiation and that it is not responsible for the action of cytokine-induced bone resorption. We have also shown evidence that osteoblasts have ecNOS which may control the basal level of osteoblastic differentiation.

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