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EFFECT OF NITRIC OXIDE ON MOUSE CLONAL OSTEOGENIC CELL, MC3T3-E1, PROLIFERATION IN VITRO

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KEY WORDS

osteoblasts; nitric oxide; proliferation; prostaglandin

ABSTRACT

Nitric oxide (NO) is a very small lipophilic molecule which rapidly diffuses and reaches the cytoplasmic components, and results in the activation of diverse biological function. It has been already reported that cultured osteoblasts synthesize NO in response to proinflammatory cytokines and lipopolysaccaride. In terms of the action of NO on bone metabolism, cytokine-induced NO by osteoblast inhibits bone resorption through inducing the apoptosis of osteoblast progenitor cells and suppressing the osteoblast activity. Also, NO synthase (NOS) inhibitor, N^G-monomethyl-L-arginine is reported to induce a dose-dependent inhibitory effect on the proliferation of osteoblast-like cell lines MG63 and ROS 17/2.8, which indicate that NO may stimulate cell proliferation. On the other hand, cytokine-induced NO is reported to reduce osteoblast activity significantly in high concentration, as was evidenced by inhibition of DNA synthesis, cell proliferation, alkaline phosphatase activity, and osteocalcin production. Thus, the effect of NO on osteoblast activities is still controversial.

In the present study, S-nitroso-N-acetyl-dl-penicillamine(SNAP), NO donor enhanced DNA synthesis of MC3T3-E1 in vitro. This activation seems to be mediated by NO directly because specific NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-0xide (carboxy-PTIO) partially attenuated the osteoblast proliferation induced by SNAP. On the other hand, the guanylate cyclase inhibitor, LY83583, failed to abolish the effect of SNAP on DNA synthesis of osteoblasts and 8-bromo cyclic guanosine 3',5'-monophosphate (cGMP), substituting for the accumulation of

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intracellular cGMP in osteoblasts, did not enhance the incorporation of ^{3}H-thymidine (^{3}H -TdR).

It is, then, suggested that osteoblast proliferation might be enhanced by NO independently apart from the activation of cytoplasmic guanylate cyclase and cGMP-dependent mechanisms.

INTRODUCTION

Nitric oxide (NO) is a very small lipophilic molecule which rapidly diffuses and reaches the cytoplasmic components, and results in the activation of diverse biological function. NO has been implicated as an important gas mediator in a biological process involving vasodilatation, inflammation and neurotransmission.

It is reported that cultured osteoblasts synthesize NO in response to proinflammatory cytokines and lipopolysaccaride [3, 20]. Cytokine-induced NO by osteoblast inhibits bone resorption through inducing the apoptosis of osteoblast progenitor cells and suppressing the osteoblast activity [29, 30]. In terms of the cell proliferation, NOS inhibitor, N^{G}-monomethyl-L-arginine is reported to induce a dose-dependent inhibitory effect on the proliferation of osteoblast-like cell lines MG63 and ROS 17/2.8, which indicate that NO may stimulate bone cell proliferation [23].

On the other hand, it is reported cytokine-induced NO significantly reduced osteoblast activity at high concentration, as was evidenced by inhibition of DNA synthesis, cell proliferation, alkaline phosphatase activity, and osteocalcin production. Thus, the effect of NO on osteoblast proliferation is diverse depending on its concentration 9, 23), However, there is no report as to whether NO induces osteoblast cell proliferation directly in vitro. Therefore, we have investigated the effect of NO-donor, SNAP, on osteoblast-like cell, MC3T3-E1 proliferation to address this issue.

MATERIALS AND METHODS

1. Osteoblast cell culture

MC3T3-E1 clonal osteogenic cell line derived from newborn C57BL/6 mouse calvariae [27] were used for these studies. Cells were cultured in alpha-minimal essential medium (a-MEM; Gibco BRL, Grand Island, NY) supplemented with 10 % heat inactivated fetal bovine serum(FBS; Biowhittaker, Walkersville, ML), 100 units/ml penicillin, 50 μg/ml streptomycin and 60 μg/ml ascorbic acid in a humidified atmosphere of 5 % $C0_2$ at 37 °C

Cells were plated in 96 multi-well plate (Corning Incorporated, Corning, NY) at a cell density of 1.0×10^{4} cells/well for evaluating cell proliferation. Immediately after cells were attached to the bottom of culture plate, monolayer cultures were subjected to the experiments described below.

2. Assay for measuring DNA. synthesis of MC3T3-E1mouse clonal osteogenic cell

In order to examine the effect of NO on MC3T3-E1 cell proliferation, we examined DNA synthesis of these cells by quantifying the radioactivity of ^{3}H-thymidine(^{3}H-TdR) incorporated into cells. Cells in 96 multi-well plate were cultured with a various concentration of S-nitroso-N-acetyl-dl-penicillamine (SNAP, CAYMAN CHEMICAL COMPANY, Denver, CO) that is one of the NO donors, for 72 hours. Fifteen hours before terminating proliferation assay of incubation time, 37 kBq of ^{3}H-TdR (DuPont/NEN Research Products, Boston, MA) were added to each well. At the end of assay, cells were washed three times with distilled water. One hundred µl of scintillation liquid were then added and cell associated ^{3}H-TdR was determined using scintillation counter (PACKARD INSTRUMENT COMPANY, Mariden, CT).

For testing the specificity of NO on these observations, MC3T3-E1 cells were cultured for 72-h with varying concentration of SNAP in the presence of NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5- tetramethylimidazoline-1-oxyl-3-0xide[1)] (carboxy-PTIO, Funakoshi Co.Ltd. Tokyo). Also, MC3T3-E1 cells were cultured with SNAP in the presence of 6-anilin0-5,8-quinolinedione [23)] (LY83583, CAYMAN CHEMICAL COMPANY, Denver, CO) , inhibitors of guanylate cyclase. Also, cells were cultured with 8-bromo cyclic GMP (8-bromo cGMP; SIGMA, St. Louis, MO) substituting for the accumulation of intracellular cGMP in order to examine the mechanisms relating to the NO-induced osteoblast cell proliferation in vitro.

3. Data analysis

Statistical analysis was carried out on all data points with regard to control by an unpaired Welch's t-test. Each data point from cell proliferation assay represented the mean of six separate samples with the corresponding standard error of the mean (SEM). P values under 0.05 were considered significant statistically.

RESULTS

Effects of NO on DNA synthesis of MC3T3-EI1 cell

In order to examine the effect of NO on proliferation of MC3T3-E1 cell, cells were cultured with NO donor, SNAP and the extent of DNA synthesis was measured. Incubation with a varying concentration of SNAP for 72 hours demonstrated that ^{3}H-TdR incorporation into MC3T3-E1 was increased in a dose dependent fashion up to 100µM SNAP (Fig.1-a). At 10µM SNAP, ^{3}H-TdR incorporation was increased by 12% compared to the control and was further increased at 100U/ml SNAP (133 % of the control). Significant augmentation of ^{3}H-TdR incorporation was observed in the presence of over 10µM of SNAP. This concentration of SNAP is reported to be equivalent to 0.01 µM of NO.

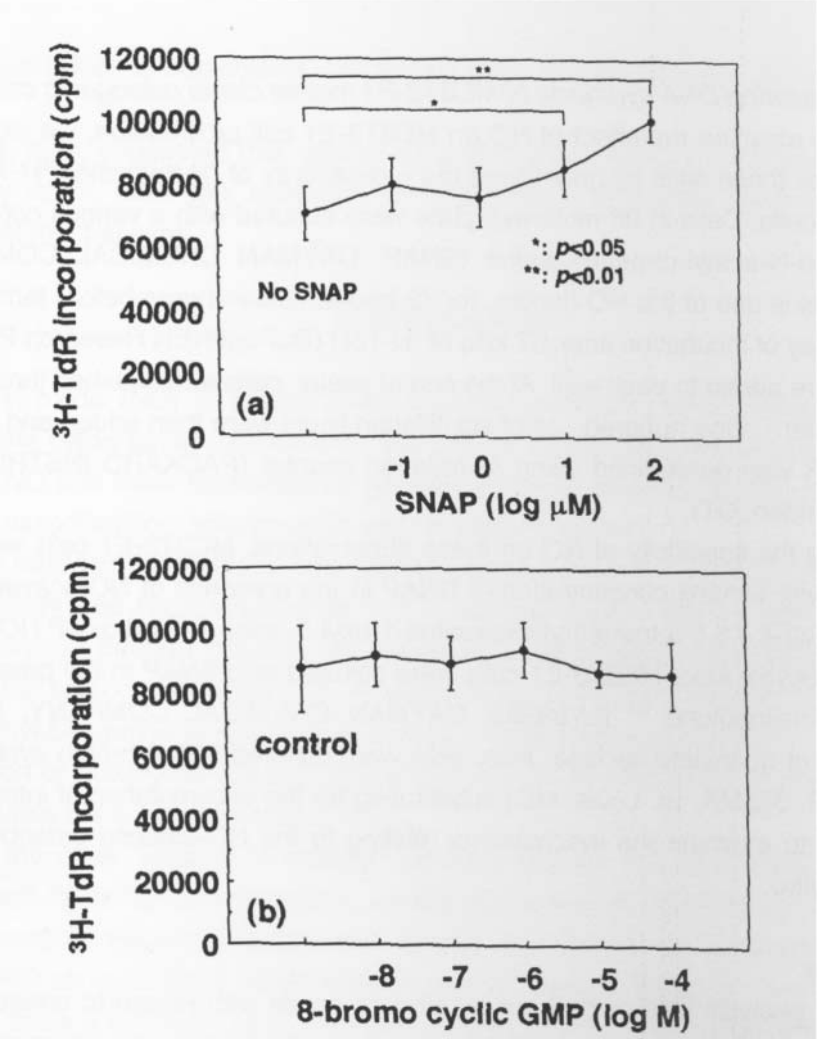

Figure 1 . Effect of (a) SNAP or (b) 8-bromo cyclic GMP on MC3T3-E1 cell proliferation. In order to examine the effect of NO on proliferation of MC3T3-E1 cell, cells were cultured with NO donor, SNAP and cell proliferation was evaluated. Also, cells were cultured with 8-bromo cGMP and ^{3}H-TdR incorporation was measured to detect whether accumulation of intracellular cGMP directly mediates MC3T3-E1 cell proliferation. ^{3}H-TdR incorporation into MC3T3-E1 cell was increased in a dose dependent manner up to 100 μM SNAP.
Significant augmentation of cell proliferation was observed in the presence of over 10 μM of SNAP ($p<0.05$). On the other hand, it was not increased by 8-bromo cGMP and no significant augmentation of cell proliferation was observed in the presence of up to 10^{-4}M 8-bromo cGMP.

	^{3}H-TdR incorporation (% of control) (cpm)		
SNAP (μM)	None	Carboxy-PTIO (10 μM)	LY83583 (10 μM)
0	100 ± 4.5 [a)]	100 ± 9.9	100 ± 9.9
	(45766 ± 2083)	(48093 ± 2573)	(46798 ± 4678)
0.1	106 ± 6.8	101 ± 8.6	104 ± 5.1
1	111 ± 10.6	106 ± 10.8	111 ± 9.6
10	121 ± 3.0 [b)]	106 ± 9.4 [d)]	120 ± 8.8 [f)]
100	130 ± 6.7 [c)]	103 ± 8.6 [e)]	129 ± 6.8 [g)]

Table. Effect of NO scavenger, carboxy-PTIO or guanylate cyclase inhibitor, LY83583 on SNAP induced cell proliferation.
When MC3T3-E1 cells were cultured with a varying concentration of SNAP in the presence of 10 μM carboxy-PTIO or LY83583. Ten μM of carboxy-PTIO attenuated the enhancement of MC3T3-E1 cell proliferation by SNAP in some extent. On the other hand, ^{3}H-TdR incorporation was not affected by addition of 10 μM LY83583. Data represent the means ± SD from six samples. a) vs. b): $p < 0.05$., a) vs. c): $p < 0.05$, b) vs. d): $p < 0.05$., c) vs. e): $p < 0.05$, c) vs. f): p = not significant., c) vs. g):p = not significant.

In this experiment, we use SNAP for substituting NO. Therefore, in order to examine whether this augmentation of ^{3}H-TdR incorporation by SNAP is due to NO directly or by anything else derived from SNAP, the effect of specific NO scavenger, carboxy-PTIO on SNAP induced the increase of DNA synthesis was investigated. When MC3T3-E1 cells were cultured with a varying concentration of SNAP in the presence of 10 μM carboxy-PTIO, it attenuated the enhancement of ^{3}H-TdR incorporation into MC3T3-E1 by SNAP in some extent. At both 10 and 100 μM SNAP, these radiouptakes were decreased to 106%and 103% of control respectively by addition of 10 μM carboxy-PTIO. These results suggest that DNA synthesis of MC3T3-E1 may be promoted by NO directly (Table).

NO is reported to activate cytoplasmic guanylate cyclase in vascular smooth muscle and decreases vascular tone through cGMP-dependent mechanism [17]. Therefore, at first, the effect of guanylate cyclase inhibitor on NO-induced MC3T3-E1 DNA synthesis was investigated. When MC3T3-E1 cells were cultured with a varying concentration of SNAP in the presence of 10 μM LY83583, it failed to abolish the effect of SNAP on MC3T3-E1 DNA synthesis in vitro. At both 10 and 100 μM SNAP, ^{3}H-TdR incorporation was not affected by addition of 10 μM LY83583 (Table).

Then, the effect of 8-bromo cGMP on MC3T3-E1 DNA synthesis were examined in order to detect whether accumulation of intracellular cGMP directly mediates MC3T3-E1 cell proliferation or not. Up to 10^{-4} M of 8-bromo-cGMP did not affect or enhance ^{3}H-TdR incorporation into MC3T3-E1 in this study (Fig.1-b). Thus, it is suggest that exogenous NO might be partly involved in cell proliferation without mediating cytoplasmic guanylate cyclase activation in MC3T3-E1 .

DISCUSSION

In accordance with previous works [20, 21], MC3T3-E1 cells produce low or undetectable amounts of NO in basal conditions. It is reported due to the limited sensitivity of Griess assay, coupled with the fact that constitutive NO synthase (cNOS) enzymes produced relatively small amounts of NO in response to defined stimuli [14, 16], thus NO is not detected without any stimulation such as cytokines or lipopolysaccarides. On the other hand interleukin-1α (lL-1α) stimulation of MC3T3-E1 cells caused a substantial increase in NO production as previously confirmed by several previous workers [9, 20, 21], which is suggested due to activation of inducible NOS (iNOS) pathway. Also, the evidence of cNOS and cytokine-induced iNOS activity in cultured osteoblast-like cells from various species have been shown. For instance, human osteoblasts constitutively produce NO through the endothelial cNOS (ecNOS) pathway, but demonstrate that this does not appear to exert an appreciable effect on osteoblast growth or differentiation under basal conditions.

It is recently well established that the effect of cytokines on bone cell activity is mediated in part by NO derived from the activation of

iNOS pathway [7]. Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 β (lL-1β) are well known as modulators of bone remodeling in vitro and in vivo. These cytokines induce the endogenous NO production in various cell types, including osteoblasts and osteoclasts, and high NO levels are correlated with the loss of cell viability [29]. NO greatly enhances the cytotoxic effect on these cells.

In terms of the osteoblast apoptosis induced by NO, it is suggested that the net loss of bone mass in inflammatory conditions is primarily caused by reducing the bone formation due to the disruption of coupling mechanism by NO [16]. High concentrations of NO are inhibitory for cells of the osteoblast lineage, and NO production appears to be partly responsible for the inhibitory effects of cytokines on osteoblast proliferation. Thus, the importance of NO as an cytotoxic mediator in the osseous microenvironment are underlined and it is proposed that this cytotoxic action of NO might explain the observed deficiency of bone formation in inflammatory sites [4] .

On the other hand, NO induced by cytokines is reported to inhibit the bone resorption through inducing apoptosis of osteoclast progenitors and suppressing osteoclast activity [29]. When the osteoclasts generated in 10-day cocultures of mouse osteoblasts and bone marrow cells were stimulated with IL-1β, TNF-α and interferon-γ (lFN-γ), NO production was markedly enhanced by 50- to 70-fold, and osteoclast formation was virtually abolished resulting in the marked inhibition of bone resorption in pit model. This superinduction of NO is largely responsible for the selective inhibitory effect of IFN-γ on cytokine-induced bone resorption.

At lower concentrations, however, NO has different effects. Moderate induction of NO potentiates bone resorption, and the constitutive production of NO at low concentrations promotes the proliferation of osteoblast-like cells and modulates osteoblast function [6]. It is also reported that a slow and moderate release of NO stimulates the replication of primary rat osteoblasts and alkaline phosphatase activity, while a rapid release and high concentrations of NO inhibit proliferation and induce apoptosis [12]. From the animal study, NO is expressed during fracture healing in rats fracture model, that suppression of NOS impairs fracture healing, and that supplementation of NO can reverse the inhibition of healing produced by NOS inhibitors [2, 5]. Thus, the biological effects of NO seems to be diversified by its concentration employed in situ, where either cNOS or iNOS may act as a predominant synthase of NO. While NO seems to be involved in catabolic processes in response to inflammation, it also mediates anabolic processes mediated by mechanical strain, sex hormones and fracture healing.

In the present study, we analyzed the DNA synthesis under NO-donor stimulated conditions in rat osteogenic cell line MC3T3-E1. As shown in Fig.1-a, SNAP has enhanced the DNA synthesis of MC3T3-E1 cells. At 10 μM of SNAP equivalent for 0.01 μM of NO, ^{3}H-TdR incorporation was increased compared to the control culture, which sug-

gest that low concentration of NO may be able to stimulate cell proliferation as reported elsewhere [12]. This activation seems to be mediated by NO directly because specific NO scavenger, carboxy-PTIO [1] partly attenuated this DNA synthesis of MC3T3-E1 induced by SNAP.

As an intracellular signaling pathway, NO is reported to activate cytoplasmic guanylate cyclase in vascular smooth muscle and decreases vascular tone through cGMP-dependent mechanism [18]. In this culture condition, the guanylate cyclase inhibitor, LY83583, failed to abolish the effect of SNAP on MC3T3-E1 cell proliferation. Also, up to 10^{-4} M of 8-bromo cGMP, which is substituting for the accumulation of intracellular cGMP did not affect or enhance ^{3}H-TdR incorporation into MC3TR-E1 . It is, therefore, speculated that osteoblast proliferation might be enhanced by exogenous NO independently apart from the activation of cytoplasmic guanylate cyclase and cGMP-dependent mechanisms.

In terms of alternative mechanism which is involved in the promotion of cell proliferation by NO, interaction between NOS and cyclooxygenase (COX) pathways in osteoblastic MC3TR-E1 cells are proposed. In MC3T3-E1 , the activation of COX pathway by NO was suggested to mediate subsequent progressive accumulation of prostaglandinE2 (PGE_2) because aminoguanidine, a selective inhibitor of iNOS, significantly suppressed the PGE_2 production by IL-1$\alpha$and TNF- α. Also, it was revealed that nitric oxide compound-18 (NOC-18), a novel NO donor, reversed this suppression and NOC-18 increased PGE_2 production by itself from MC3T3-E1 [11]

The effects of PGE_2 on fracture repair were reported in many studies. The long term subcutaneous injection of PGE_2 increases the metaphyseal bone masses in rats [10]. The oral administration of PGE_2 on a rib fracture in beagle alters the phase of the regional remodeling cycle [25]. In addtion, local infusion of PGE_2 at the osteotomy site of a plated tibia has been shown to stimulate callus formation in rabbits[28]. In vitro, PGE_2 has shown to in crease collagen synthesis in bone organ cultures [15, 19] and stimulate proliferation of some osteoblastic cells [8, 13]. Thus, PGE_2 is considered to be an important regulator of bone formation and these reports suggested that the possibility of PGE_2 induction by NO might explain our observation, though the detail mechanism of this augmentation of cell proliferation is still unclear.

In conclusion, NO plays an important role in the bone remodeling through modification of cell proliferation. An effort should be employed to elucidate the detail mechanisms of the MC3T3-E1 cell proliferation induced by NO.

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