

## Effect of Sodium Nitroprusside on the Activation of Mouse Osteoblastic Cells

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*Nitric oxide (NO), which is produced from arginine by a nitric oxide synthase, is a short-lived free radical that plays crucial role in a variety of tissues. Recently, it has been reported that NO is produced by osteoblast stimulated by lipopolysaccharide and several cytokines. Although NO appears to inhibit osteoclastic differentiation and activation, little is known about its possible role in osteoblastic function. Therefore, the effect of sodium nitroprusside (SNP), as a donor of nitric oxide, on osteoblastic activation in terms of alkaline phosphatase (ALP) activity and calcified nodule formation in the osteoblastic cells were studied. SNP increased not only the ALP activity but also the calcified nodule formation. ALP activity was enhanced significantly by the addition of SNP (30-300  $\mu$ M) with 14-31% magnitude when compared with control. SNP also stimulated calcium phosphate - containing calcified matrix formation. The number of calcified nodules was increased significantly by continuous treatment of cultures with 30  $\mu$ M SNP for 21 days. In addition to the recently reported inhibition of osteoclast function by NO, these results raise the possibility that NO could promote osteoblastic bone formation by stimulating osteoblastic function and mineralization as well.*

**Key words:** Osteoblast, Nitric oxide, Sodium nitroprusside, Alkaline phosphatase, Calcified nodule, Bone formation

### Introduction

Osteoblasts, which are primarily responsible for bone formation, are derived from undifferentiated mesenchymal cells. They secrete a number of phenotype - specific macromolecules such as osteocalcin (Price, 1983), type I collagen and a wide variety of biomolecules, which affect on the regulation of cell division, cell differentiation and bone mineralization. Since bone is a complex tissue which consists of a number of cell types, biomolecules produced by one of these cell types may affect on the regulation of cellular function of them. It has been widely accepted that a large number of factors, such as hormones, cytokines, or growth factors, are related to the bone metabolism which is characterized by the coupling of osteoclast - mediated bone resorption and osteoblast - mediated bone formation. In the past few years, besides these factors, nitric oxide (NO) has been postulated to be an important regulating factor in the process of bone metabolism.

Nitric oxide, which is produced from arginine by nitric oxide synthase (NOS), has been found in various cells and tissues: vascular system (Moncada *et al.*, 1987; Ignarro *et al.*, 1987; Palmer, 1988), macrophage (Tayeh and Marletta, 1989;

Kwon *et al.*, 1989; Albina *et al.*, 1989a, 1989b; Stuehr and Nathan, 1989; Gaillard *et al.*, 1991), neutrophil (McCall *et al.*, 1989), mast cell (Masini *et al.*, 1991), nervous system (Bredt and Snyder, 1989; Förstermann *et al.*, 1990), and gastric mucosa (Whittle *et al.*, 1990; Brown *et al.*, 1992). Currently, NO can be considered as a mediator of signalling pathways in these tissues.

Recently, Damoulis and Hauschka (1994) and Riancho *et al.* (1995) have presented evidence that mouse osteoblasts can be induced to produce NO by cytokines and bacterial endotoxin, which in turn may have a role in regulating bone remodeling and in bone pathophysiology. Since several evidences have suggested that osteoblasts are involved in the bone resorption as well as bone formation, NO produced by osteoblasts may act on osteoclasts and osteoblasts as well. Moreover, it has become evident that NO exerts a powerful inhibitory effect on the bone resorbing activity of the osteoclasts. It has been shown that NO act directly on the osteoclast to produce a shape changes which were associated with a inhibition of bone resorption (MacIntyre *et al.*, 1991) and that there are indications of an arginine - dependent NO pathway in osteoclasts (Kasten *et al.*, 1993). These reports postulate that NO produced

by osteoblasts may act on osteoblasts as autocrine and osteoclasts as paracrine function in the process of bone remodeling. Although many previous studies were focused on the possibility that NO appears to inhibit osteoclastic bone resorption, little is known about its possible role in osteoblastic function. The present study was undertaken to investigate whether NO is implicated in osteoblastic activation and bone formation in terms of alkaline phosphatase activity and calcified nodule formation.

## Materials and Methods

### Materials

All culture media were purchased from GIBCO BRL Laboratories (Grand Island, NY, USA). All disposable culture wares were purchased from Corning Incorporated (Corning, NY, USA). All other chemicals used were, unless other stated, purchased from Sigma (St. Louis, MO, USA) as highest quality available.

### Culture of MC3T3-E1 cells

An osteoblastic cell line MC3T3-E1 established by Suda *et al.* were kindly provided by Dr. Peter Hauschka (Children's Hospital, Boston, MA, USA). The cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) supplemented with 10% fetal bovine serum ( $\alpha$ -MEM/FBS) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

### Measurement of alkaline phosphatase(ALP) activity

MC3T3-E1 cells were plated into 96-well plate at a density of  $2-3 \times 10^3$  cells/well in  $\alpha$ -MEM/FBS and cultivated until confluency was obtained. Then the cells were treated with appropriate concentration of sodium nitroprusside (SNP, 0-300  $\mu$ M) in  $\alpha$ -MEM supplemented with 0.4% FBS ( $\alpha$ -MEM/0.4% FBS) for 48 hours. After removal of the culture media from the wells, the cells were washed with Dulbecco's phosphate buffered saline and treated with 0.1% Triton X-100/saline for 30 min. To determine the ALP activity, resultant cell lysate was incubated in 0.1 M glycine-NaOH buffer (pH 10.4) with 100 mM p-nitrophenyl phosphate as a substrate at 37°C for 10 min. The optical den-

sity of p-nitrophenol, a reaction product, was read at 405 nm using ELISA reader (SLT Lab-instruments, 400 SFC) spectrophotometrically. To determine the time - course of ALP activity, the cells were cultured in the presence or absence of SNP (30  $\mu$ M) and measured ALP activity after 24 and 48 hours as same method as described above.

### Nitrite assay

MC3T3-E1 cells were plated into 96-well plate at a density of  $2-3 \times 10^3$  cells/well in  $\alpha$ -MEM/FBS and cultivated until confluency was obtained. Then the cells were treated with SNP (0-300  $\mu$ M) in phenol red-free  $\alpha$ -MEM/0.4% FBS for 48 hours. NO synthesis was assessed by measuring the nitrite anion concentration, a breakdown product of NO, in the cell culture medium. One hundred microliter of cultured medium was mixed with an equal volume of Griess reagent (0.5% sulfanilamide, 0.05% naphthylethylene diamine, 2.5% phosphoric acid), incubated for 15 min, and the optical density was determined at 530 nm using ELISA reader.

### Calcified nodule formation

MC3T3-E1 cells were plated into 24-well plate at a density of  $1-2 \times 10^4$  cells/well in  $\alpha$ -MEM/FBS and cultivated until reached confluency. After confluency, media were changed with  $\alpha$ -MEM/FBS supplemented with 50  $\mu$ g/ml ascorbic acid and 10 mM  $\beta$ -glycerophosphate. Media were changed every second or third day and cultures were maintained up to 21 days in the presence or absence (control) of SNP (10-100  $\mu$ M). To observe the produced calcified nodule, cell layer was fixed with neutral buffered formalin and stained in situ by the von Kossa technique for mineral deposits. Number of mineralized nodules were counted at 40 $\times$  magnification using a light microscope.

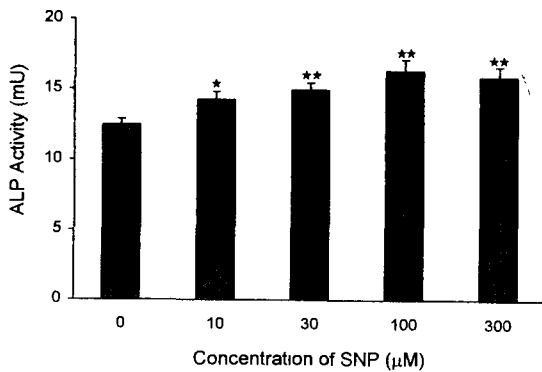
### Statistical analysis

Data were expressed as mean  $\pm$  S.E. of 5 replicates. The statistical significance of the difference was determined by a paired Student's t-test.

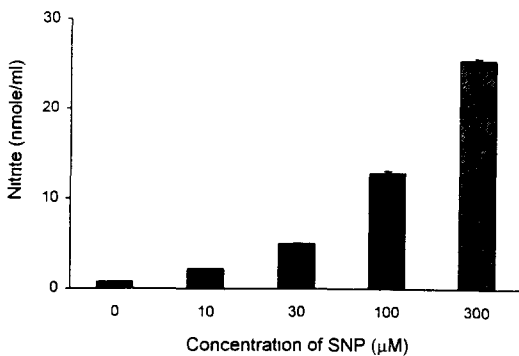
## Results

### Effect of SNP on the ALP activity

To determine whether SNP affects on the ALP ac



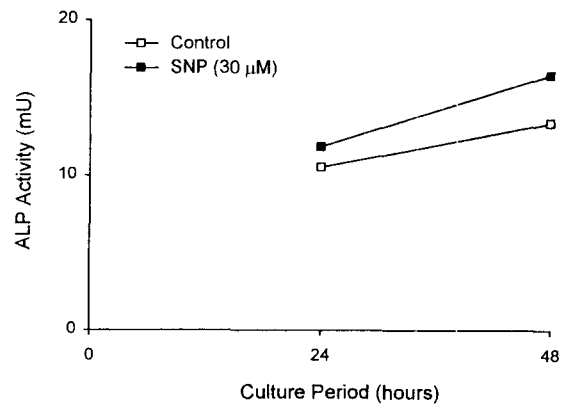
**Fig. 1.** Effect of SNP on the ALP activity of MC3T3-E1 cells in culture. MC3T3-E1 cells were cultured in the presence or absence of SNP for 48 hours. ALP activity was assayed spectrophotometrically using PNPP as a substrate. One unit(U) of ALP activity was defined as amount of enzyme which catalyzes the transformation of 1 µmole of PNPP per min at 37°C. Data represent Mean ± S.E. of 5 replicates. \* Significantly different from control,  $p < 0.05$  \*\* Significantly different from control,  $p < 0.01$ .



**Fig. 2.** Change in nitrite formation of cultured medium of MC3T3-E1 by addition of SNP. MC3T3-E1 cells were cultured in phenol red-free media with SNP for 48 hours. Nitrite of the cultured medium was assayed with Griess reagent. Data represent Mean ± S.E. of 5 replicates.

tivity of MC3T3-E1 cells, the cells were treated with appropriate concentration of SNP (0-300 µM) in  $\alpha$ -MEM/0.4% FBS for 48 hours. Significant change in ALP activity was observed when MC3T3-E1 cells were treated with SNP for 48 hours. As seen in Fig. 1, SNP produced a stimulatory effect on ALP activity with 14-31% magnitude when compared with control.

Fig. 3 shows a typical time course of change in ALP activity in culture of MC3T3-E1 cells in the presence (30 µM) or absence of SNP. ALP activity was increased by the addition of SNP in both of the time point, 24 (12%) and 48 hours (23%). The differences between control and SNP-treated group was bigger at a later stage of the culture period.



**Fig. 3.** Time-course of ALP activity in MC3T3-E1 cells in culture. MC3T3-E1 cells were cultured in the presence or absence of SNP. ALP activity was assayed after 24 and 48 hours. Data represent Mean ± S.E. of 5 replicates.

### Nitrite assay

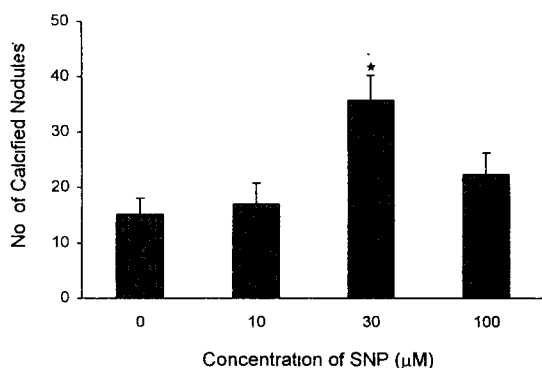
To verify the existence of nitrite ( $\text{NO}_2^-$ ), a stable metabolite of NO, by the addition of SNP, MC3T3-E1 cells were treated with SNP (0-300 µM) in phenol red-free  $\alpha$ -MEM/0.4% FBS for 48 hours. When treated with SNP, MC3T3-E1 cells have the ability to release significant amounts of NO, which result in dose-dependent  $\text{NO}_2^-$  accumulation in the culture medium during 48 hours of culture (Fig. 2).

### Effect of SNP on the calcified nodule formation

To investigate the effect of SNP on the calcified nodule formation, MC3T3-E1 cells were maintained up to 21 days with treatment of SNP (10-100 µM). The number of calcified nodules was increased by treatment of cultures with SNP continuously for 21 days, and also considerable number of calcified nodules were formed in control group of MC3T3-E1 cells in culture for 21 days (Fig. 4).

### Discussion

Recently, it has been reported that NO is produced by osteoblasts (Damoulis and Hauschka, 1994) and articular chondrocytes (Stadler *et al.*, 1991). These reports indicate that NO produced by osteoblasts and chondrocytes might regulate the activity of neighboring cells. In these regards, NO might participate in the regulation of bone remodeling. And also Löwik *et al.* (1994) proposed that the NO production by osteogenic cells (osteoblasts and chondrocytes) may represent an im-



**Fig. 4.** Effect of SNP on the calcified nodule formation by MC3T3-E1 cells in culture. MC3T3-E1 cells were maintained in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for 21 days. Each concentration of SNP was added simultaneously during the culture period. Data represent Mean ± S.E. of 5 replicates. \*Significantly different from control,  $p < 0.05$ .

portant regulatory mechanism of osteoclastic activity under pathological conditions characterized by release of bone - resorbing inflammatory cytokines.

Since osteoclasts and osteoblasts are closely related in the process of bone remodeling with a highly ordered sequence of bone resorption and formation, most of the previous works have focused on the possibility that NO regulates the activity of osteoclastic bone resorption. Whereas, little is known about its possible role in osteoblastic function. Although it is generally accepted that osteoblasts release various factors which modulate osteoclast activity, the transcellular communication between osteoblasts and osteoclasts is limited by the short half-life of NO. In this regard, we hypothesized that NO produced by osteoblasts may be involved in the osteoblastic activity as an autocrine manner.

Osteoblasts have receptors for parathyroid hormone on their cell surface (Silve *et al.*, 1983) and produce type I collagen and noncollagenous proteins as well as a wide variety of biomolecules which affect on the regulation of cell division, cell differentiation and bone mineralization. ALP activity and osteocalcin are classical marker for the osteoblastic phenotype.

Since SNP causes a discernible decrease in systolic blood pressure, it has been used to treat the hypertensive patients. In this experiment, the existence of nitrite ( $\text{NO}_2^-$ ) was confirmed by the addition of SNP to the MC3T3-E1 cells in phenol red-free α-MEM supplemented with 0.4% FBS for 48 hours. When treated with SNP, MC3T3-E1 cells have the ability to release significant amounts of

NO, which result in  $\text{NO}_2^-$  accumulation in the culture medium dose-dependently during 48 hours of culture. SNP has been shown to be a potent donor of NO, therefore, SNP may be used to determine to what extent osteoblastic functions are modulated by NO. SNP stimulated the ALP activity and calcified nodule formation in the present study. These results suggest that the NO is involved in the osteoblastic bone formation. It has been recently reported by Fox and co-workers (1995) that bone formation induced by mechanical stimulation is due to activation of a pre-existent NOS in cells of the osteoblasts. And they found that administration of NOS inhibitor completely prevented the increase in bone formation induced by mechanical stimulation. Pitsillides *et al.* (1995) have reported similar experimental findings in that mechanical strain results in a rapid, transient NO release from isolated osteoblast-like cells, osteocytes, and from rat ulnae. These findings suggest that the loading - related adaptive changes in bone cell behavior may be mediated at least in part by NO.

NO seems to show stimulatory effects on osteoblastic bone formation in terms of activation of ALP activity and calcified nodule formation by treatment of SNP in culture of MC3T3-E1 cells. ALP activity of MC3T3-E1 cells was enhanced by 30-300 μM SNP, and significant change was observed when MC3T3-E1 cells were treated with SNP for 48 hours. ALP activity was increased by the addition of SNP in both of the time point, 24 (12%) and 48 hours (23%), and the differences between control and SNP-treated group was bigger at a later stage of the culture period.

Another interesting finding was obtained with respect to calcified nodule formation in long-term culture of MC3T3-E1 cells. The number of calcified nodules was increased significantly by the addition of 30 μM SNP. Molecular mechanisms which are involved in the stimulatory action of SNP on ALP activity and calcified nodule formation remain to be elucidated.

In conclusion, although the precise action of NO remains to be elucidated in detail, the stimulatory action of NO in ALP activity and calcified nodule formation in this study seems to be associated, at least in part, with the osteoblastic bone formation.

## Conclusion

The present study was performed to investigate

the effects of nitric oxide on the osteoblastic activation and bone formation. To determine whether SNP affects on the ALP activity of MC3T3-E1 cells, the cells were treated with appropriate concentration of SNP (0-300  $\mu$ M) in  $\alpha$ -MEM supplemented with 0.4% FBS for 48 hours. To determine the time-course of ALP activity, the cells were cultured in the presence or absence of SNP (30  $\mu$ M) and measured ALP activity after 24 and 48-hours culture. To confirm the existence of nitrite ( $\text{NO}_2^-$ ) by the addition of SNP, MC3T3-E1 cells were treated with SNP (0-300  $\mu$ M) in phenol red-free  $\alpha$ -MEM supplemented with 0.4% FBS for 48 hours. NO synthesis was assessed by measuring the nitrite anion concentration in the culture medium by Griess reagent. To investigate the effect of SNP on the calcified nodule formation, MC3T3-E1 cells were maintained in  $\alpha$ -MEM supplemented with 50  $\mu$ g/ml ascorbic acid and 10 mM  $\beta$ -glycerophosphate up to 21 days with treatment of SNP (10-100  $\mu$ M). Calcified nodules were stained *in situ* by the von Kossa technique. Number of mineralized nodules were counted under light microscope.

Significant change in alkaline phosphatase activity was observed when MC3T3-E1 cells were treated with SNP for 48 hours. SNP produced a stimulatory effect on ALP activity with 14-31% magnitude when compared with control. ALP activity was increased by the addition of SNP in both of the time point, 24 (12%) and 48 hours (23%). The differences between control and SNP-treated group was bigger at a later stage of the culture period. When treated with SNP, MC3T3-E1 cells have the ability to release significant amounts of NO, which result in  $\text{NO}_2^-$  accumulation in the culture medium dose-dependently during 48 hours of culture. The number of calcified nodules was increased by treatment of cultures with 30  $\mu$ M SNP continuously for 21 days.

These findings demonstrate that NO could promote osteoblastic bone formation by stimulating osteoblastic function and mineralization.

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