Nitric oxide induces osteoblast apoptosis through the de novo synthesis of Bax protein [☆]

Ruei-Ming Chen [a], Hwa-Chang Liu [b,*], Yi-Ling Lin [a], Wen-Chyi Jean [b], Jui-Shui Chen [b], Jyh-Horng Wang [b]

[a] Department of Anesthesiology, Taipei Medical College, Wan-Fang Hospital, No. 111, Sec. 3, Hsing-Lung Road, Taipei 116, Taiwan, ROC
[b] Department of Orthopedic Surgery, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei 100, Taiwan, ROC

Abstract

Nitric oxide (NO) plays a crucial role in the physiological and pathophysiological regulations of osteoblast functions. This study is designed to evaluate the toxic effects of NO released by sodium nitroprusside (SNP), an NO donor, on neonatal Wistar rat calvarial osteoblasts from the analyses of cell viability, alkaline phosphatase (ALP) activity, cell morphology, apoptotic cells, terminal deoxynucleotidyl transferase-mediated dUTP nick end-label (TUNEL) assay, DNA ladder, and immunocytochemistry and Western blot for proapoptotic Bax protein. SNP increased the levels of nitrite, an oxidative product of NO, in the culture medium of osteoblasts in concentration- and time-dependent manners, and altered cell morphologies to round and shrinkage shapes. Administration of osteoblasts with SNP resulted in concentration- and time-dependent decreases of cell viability and ALP activity. Analysis of apoptotic cells revealed that SNP increased the percentages of osteoblasts processing apoptosis. Analyses of TUNEL and DNA ladder showed that SNP caused DNA fragmentation. Pretreatment with cycloheximide, an inhibitor of protein synthesis, partially blocked SNP-induced osteoblast apoptosis. Imunocytochemical and immunoblotting analyses revealed that SNP increased Bax protein in osteoblasts. This study suggests that SNP could increase the levels of NO in osteoblasts, and cause osteoblast apoptosis possibly through the de novo synthesis of proapoptotic Bax protein. © 2002 Orthopaedic Research Society. Published by Elsevier Science Ltd. All rights reserved.

Introduction

Osteoblasts play an important role in bone metabolism. One of the primary functions of bone tissues is structural support. Bone structure is maintained by bone remodeling, a process that is characterized by the coupling of osteoblast-mediated bone formation and osteoclast-mediated bone resorption [32]. Imbalance in the remodeling process would lead to pathophysiological diseases such as osteoporosis and osteoarthritis [9]. Varieties of systemic and local mediators modulate osteoblast or osteoclast metabolism and contribute to regulation of bone remodeling [9,13].

Nitric oxide (NO) is a gaseous free radical synthesized from L-arginine by calcium-dependent constitutive or calcium-independent inducible NO synthases [21,28,38].

The diatomic free radical has been implicated as an important regulator of vaso-regulation, neuronal transmission, immune response, and cell apoptosis [3,38]. NO is detectable in control osteoblasts and is also inducible after treatment of proinflammatory cytokines, mechanical stress, or fluid flow [10,14,19,34–36,39]. NO has the potential role to modulate proliferation and differentiation of osteoblasts and bone resorption activity of osteoclasts, and to mediate the effects of proinflammatory cytokines and mechanical stress. The free radical is able to regulate bone maintenance and remodeling [5,8,15,22]. In an animal model of inflammation-induced osteoporosis, NO is induced and associated with the reduction of osteoblast numbers, the increase of osteoclast numbers, and the decrease of bone mineral density [1]. The induction of NO in osteoblasts following treatment with proinflammatory cytokines promotes cell death [11,27]. However, the death mechanism induced by NO is still little known.

This study is designed to evaluate the cytotoxic effects of NO on osteoblasts. Sodium nitroprusside (SNP) is an NO donor used to be the source of NO. The use of NO

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donors has biochemical advantages because the donors permit the investigation of NO's role in signaling transduction pathways without interfering with NOS-involved second messenger systems. SNP is a clinical medicine that is decomposed to NO under the presence of biological systems, reducing agents or visible light [2]. Administration of osteoblasts with SNP has been reported to affect osteoblastic metabolism [31]. To evaluate the toxic effects of NO on osteoblasts, the bone cells are prepared from neonatal Wistar rat calvariae and treated with SNP. The cytotoxic mechanism is determined from the aspects of the cells' morphologies, ALP activity, cell viability, apoptotic assay, DNA fragmentation, and immunocytochemical and immunoblotting analyses for proapoptotic Bax protein.

Materials and methods

Osteoblast preparation, culture, SNP treatment and NO determination

Osteoblasts were prepared from 3-day-old Wistar rat calvariae following the sequentially enzymatic digestion method as described previously [33]. The primary osteoblasts were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, BRL, Grand Island, NY, USA) supplemented with a 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin in 250 ml polystyrene tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2. SNP (Sigma, St. Louis, MO, USA) was dissolved in a PBS buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) to 200 mM as a stock solution, stored at −20°C and protected from light for use in related experiments. Osteoblasts were treated with various concentrations of SNP for different time intervals. Osteoblast morphologies were observed and photographed by a reverse-phase microscope. The amounts of nitrite in the culture medium of osteoblasts were determined by the protocol provided in the Bioxytech NO assay kit (OXIS International, Portland, OR, USA).

Assays of cell viability and ALP activity

After treatment with SNP, osteoblast viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described previously [4]. ALP activity of osteoblasts was determined by detecting the formation of p-nitrophenol, a product of p-nitrophenyl phosphate catalyzed by ALP, following the colorimetric procedure provided in Sigma Diagnostics Alkaline, Acid and Prostatic Acid Phosphotase kit (Sigma Diagnostics, St. Louis, MO, USA).

Analysis of apoptotic cells

Using a flow cytometer, apoptotic osteoblasts were determined to detect DNA fragments in nuclei stained by propidium iodide according to the method of Nicoletti et al. [29]. After treatment, osteoblasts were harvested and fixed in cold 80% ethanol. Following a process of centrifugation and washing, the fixed cells were stained with propidium iodide and analyzed using a FACScan flow-cytometer (FACS Calibur, Becton Dickinson, San Joes, CA, USA) on the basis of a 560 nm dichroic mirror and a 600 nm band pass filter.

TUNEL assay

After treatment, untreated and SNP-treated osteoblasts were harvested, and DNA fragmentation in osteoblastic nuclei was detected following the protocol of Oncogene Research Products TdT-FragEL™ DNA Fragmentation Detection Kit (Oncogene Research Products, MA, USA).

DNA ladder analysis

Genomic DNA from control and SNP-treated osteoblasts were analyzed by a classical DNA electrophoresis method for the determination of the DNA ladder. Following treatment, osteoblasts were harvested and lysed with a lysis buffer (5% sarcosyl, 10 mM Tris–Cl, 10 mM EDTA and 20 units of proteinase K) at 50°C overnight. The rat osteoblast lysate was treated with 10 μg DNase-free RNase for 1 hour, and then was extracted with phenol-chloroform solution several times. The water layer was electrophoretically separated in a 1.2% agarose gel containing 0.1 μg/ml ethedium bromide. The DNA bands were visualized and photographed under UV-light exposure.

Immunocytochemical analysis

Control and SNP-treated osteoblasts were harvested for immunocytochemical analysis of Bax protein following the standard protocol provided by the VECTASTAIN ABC kit (Vector Laboratories, CA, USA). After treatment, osteoblasts were harvested, and spun to microslides using a cytospin-3 (Shandon Scientific Limited, Cheshire, England). The slides were fixed in 80% ethanol, incubated with 1% normal fetal bovine serum for 20 min, and reacted with the mouse monoclonal antibody against rat Bax protein, which is diluted 1:100 in TBS buffer (10 mM Tris–Cl pH 7.5, 100 mM NaCl, 0.1% Tween 20) for another 2 h. After washing, the slides were incubated with a mouse IgG antibody conjugated with horseradish peroxidase that is diluted 1:100 at room temperature for 45 min. The slides were colorimetric after reacting with 3,3'-diaminobenzidine and hydrogen peroxide.

Immunoblotting analysis

Sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) and protein transfer were carried out following the method of Chen et al. [7]. Immunodetection of Bax protein was determined using a mouse monoclonal antibody against rat Bax protein (R&D Systems, MN, USA). The intensities of protein bands in the Western blots were quantified with the aid of an IS-1000 Digital Imaging system (Alpha Innotech, San Leandro, CA, USA) as described elsewhere [6].

Statistical analysis

The statistical significance of difference between control- and drug-treated groups was evaluated by Student's t-test. A P value <0.05 was considered statistically significant. The difference between drug-treated groups was considered to have a statistical significance when a P value of Duncan Multiple Rang test was less than 0.05.

Results

Nitrite, an oxidative product of NO, was detectable in untreated osteoblasts, and exposure to 0.5, 1.0, 1.5 and 2.0 mM SNP for 16 h resulted in 41%, 63%, fivefold and sixfold increases of nitrite, respectively (Table 1). Administration of osteoblasts with 1.5 and 2 mM SNP caused 29% and 58% osteoblast death, respectively, but SNP at <1 mM was not cytotoxic to the cells. SNP at 0.5, 1.0, 1.5 and 2 mM decreased 24%, 52%, 55% and 59% ALP activity in osteoblasts, respectively.

Nitrite in the culture medium of osteoblasts pretreated with 2 mM SNP for 2, 4, 8, 16 and 24 h was augmented by about 2-, 3-, 5-, 6- and 8-folds, respectively (Table 2). Treatment of osteoblasts with 2 mM SNP for 8, 16 and 24 h caused 18%, 58% and 69% cell death, respectively. ALP activity of osteoblast exposed to 2 mM SNP for 4, 8, 16 and 24 h was reduced by 16%,

Table 1
Concentration-dependent effects of SNP on nitrite production osteoblastic viability and ALP activity

SNP (mM)	Nitrite (μM)	Cell viability (OD at 570 nm)	ALP activity (OD at 410 nm)
0	3.2 ± 0.6	0.86 ± 0.12	0.63 ± 0.08
0.1	4.0 ± 0.7	0.80 ± 0.09	0.53 ± 0.07
0.5	4.5 ± 0.4*	0.91 ± 0.11	0.48 ± 0.04*
1.0	5.2 ± 0.8*	0.95 ± 0.07	0.30 ± 0.07*
1.5	14.9 ± 3.6*	0.61 ± 0.06*	0.28 ± 0.06*
2.0	18.6 ± 5.2*	0.36 ± 0.05*	0.26 ± 0.02*

Osteoblasts were treated with 0, 0.1, 0.5, 1.0, 1.5 and 2.0 mM SNP for 16 h. The levels of nitrite in culture medium were assayed by the Griess reaction. Osteoblastic viability and ALP activity were determined using colometric methods as described in Materials and Methods. Each value was represented Mean ± S.E. for $n = 6$.

* Value significantly different from the respective control, $P < 0.05$.

Table 2
Time-dependent effects of SNP on nitrite production osteoblastic viability and ALP activity

Time (h)	Cell viability (OD at 570 nm)	ALP activity (OD at 410 nm)	Nitrite (μM)
0	1.03 ± 0.09	0.75 ± 0.06	2.6 ± 0.7
2	0.96 ± 0.02	0.73 ± 0.06	5.3 ± 0.6*
4	0.98 ± 0.02	0.46 ± 0.02*	8.1 ± 1.4*
8	0.84 ± 0.03*	0.41 ± 0.01*	13.8 ± 2.1*
16	0.43 ± 0.02*	0.32 ± 0.02*	16.5 ± 1.9*
24	0.32 ± 0.02*	0.25 ± 0.03*	21.4 ± 3.2*

Osteoblasts were treated with 2 mM SNP for 0, 2, 4, 8, 16 and 24 h. Viability and ALP activity of osteoblasts were determined using colometric methods as described in Materials and Methods. The levels of nitrite in culture medium were detected by the Griess reaction. Each value was represented Mean ± S.E. for $n = 6$.

* Value significantly different from the respective control, $P < 0.05$.

25%, 50% and 70% respectively decreases of ALP activities, respectively.

Osteoblast morphologies were observed and photographed following treatments of 2 mM SNP for 2, 4, 8, 16 and 24 h. In the 4 h-treated group, some osteoblasts' morphologies were altered by SNP (Fig. 1). In the 8 h-treated group, rounded and floated osteoblasts were markedly observed. In the 16 h-treated group, 50% osteoblasts became shrunk, rounded and floated. In the 24 h-treated group, almost 80% of the osteoblasts were detached and floated.

Exposure of osteoblasts to 1.5 and 2 mM SNP for 16 h led to 27% and 65% cells undergoing apoptosis, respectively (Table 3). The percentages of apoptotic cells in osteoblasts pretreated with 2 mM SNP for 4, 8, 16 and 24h were increased by 2.5%, 7.3%, 61% and 71%, respectively. There was no statistical difference between the control and 2 h-treated groups. TUNEL assay revealed that SNP caused DNA fragmentation in osteoblastic nuclei (Fig. 2). Genomic DNA from the control and SNP-treated osteoblasts were isolated and

electrophoretically separated in agarose gel (Fig. 3). Administration of osteoblasts with SNP caused 400 base-pair fragments of the DNA ladder, but the effect was not observed in untreated osteoblasts (lanes 2 and 3).

Exposure of osteoblasts to cycloheximide, an inhibitor of protein translation, did not affect cell apoptosis (Fig. 4). Pretreatment with cycloheximide for 30 min and then co-treated with SNP partially blocked SNP-induced osteoblast apoptosis. Immunocytochemical analysis using a mouse monoclonal antibody against rat Bax protein showed that SNP increased the levels of Bax protein in osteoblasts with brown signals (Fig. 5, top panel, B and D). In comparison, control osteoblasts presented blue signals (Fig. 5, top panel, A and C). Immunoblotting analysis revealed that SNP caused a fivefold increase in the intensity of Bax protein (Fig. 5, bottom panel).

Discussion

Administration of SNP increases the levels of NO in osteoblasts and further causes oxidative stress to the bone cells. SNP is a clinical medicine used as a vasodilator for vasocardiac diseases because the NO donor can be decomposed to NO, a messaging molecule for relaxation of blood vessels [2]. The detailed mechanism of SNP decomposition to NO is still unknown. Several lines of evidence have reported that the NO donor can be decomposed to NO under the presence of biological systems, reducing agents or visible light [2,20]. NO has a short half-life to be easily oxidized to nitrite and nitrate [37]. The present study reveals that the levels of nitrite in rat osteoblasts were concentration- and time-dependently augmented following SNP treatment. NO is a reactive oxygen species, whose increase of the free radical will result in the increase of oxidative stress on osteoblasts and the further modulation of osteoblastic physiology or pathophysiology.

NO could regulate osteoblast survival and metabolism. In parallel to the increase of NO in osteoblasts, SNP causes concentration- and time-dependent decreases of osteoblast viability. Osteoblasts play a crucial role in bone formation. Suppression of osteoblast viability or proliferation would interfere with the dynamic balance of bone remodeling, and lead to pathophysiological conditions of bone tissues [9]. Previous studies reveal that NO, over-induced by proinflammatory cytokines, has a pathogenic role when it acts as a mediator of these cytokines for the stimulation of osteoblast death [22,27]. This study shows that NO released by an extracellular NO donor also has the similar cytotoxic effects on osteoblasts as intracellular NO induction. ALP is a marker enzyme for osteoblast metabolism [9]. Treatment of osteoblasts with SNP results in
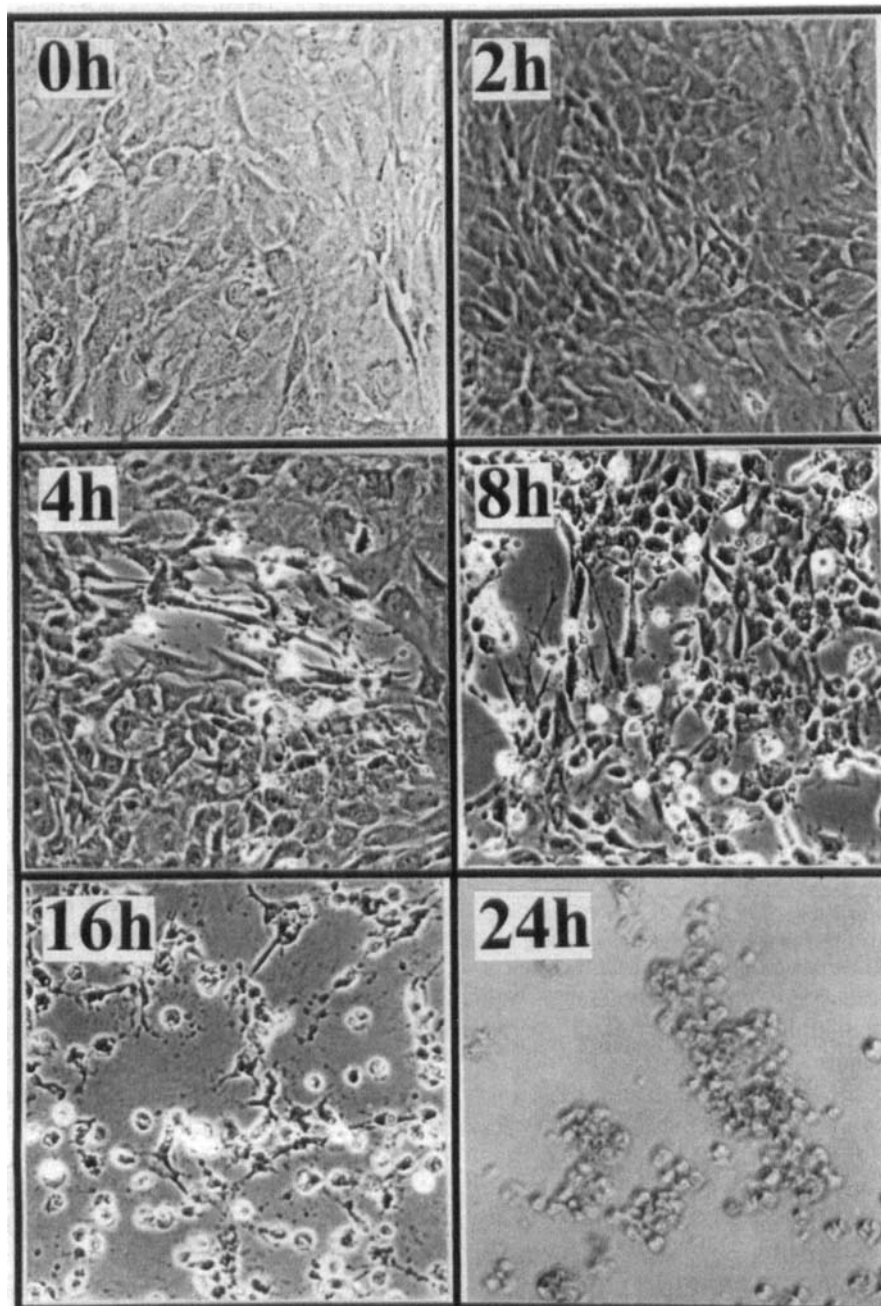
Fig. 1. Effects of SNP on alternations of osteoblast morphologies. Osteoblasts prepared from neonatal Wistar rat calvariae were treated with 2 mM SNP for 0, 2, 4, 8, 16 and 24 h. Cell morphologies were observed and photographed using a reverse phase-contrast microscopy (100×).

concentration- and time-dependent decreases of ALP activity. The reduction of ALP activity caused by NO may be partially due to the cytotoxic effects of NO on osteoblasts. McPherson et al. [23] reported that the NO donors SNP and SNAP only at high concentrations (>0.1 mM) were able to inhibited cell viability and ALP activity. The present data has the similar results that low concentrations of SNP (<0.1 mM) were not cytotoxic to osteoblasts, however, SNP at high concentrations would modulate osteoblast metabolism and lead to cell death.

There are several lines of evidence presented here to demonstrate that NO induces osteoblast death mainly through the apoptotic signaling pathway. Apoptosis, also known as programmed cell death, is an energy-dependent model of cell death. Cells processing apoptosis possess certain characteristics, including typically morphological alternation, as well as chromatin condensation and cleavage [25,26]. From morphological observations, the present study has shown that administration of SNP changes osteoblast shapes into shrun-

Table 3
Concentration- and time-dependent effects of SNP on osteoblast apoptosis

Apoptotic cells (%)			
SNP (mM)		Time (h)	
0	1.5 ± 0.3	0	1.3 ± 0.4
0.1	1.0 ± 0.1	2	1.5 ± 0.4
0.5	0.9 ± 0.2	4	$2.5 \pm 0.3^*$
1.0	1.8 ± 0.4	8	$7.3 \pm 1.7^*$
1.5	$26.5 \pm 6.9^*$	16	$60.7 \pm 9.3^*$
2.0	$65.1 \pm 5.8^*$	24	$70.6 \pm 2.5^*$

Osteoblasts were treated with 0, 0.1, 0.5, 1.0, 1.5 and 2.0 mM SNP for 16 h or with 2 mM SNP for 0, 2, 4, 8, 16 and 24 h. The percentages of apoptotic cells in osteoblasts were determined by a flow cytometric method as described in Materials and Methods. Each value was represented Mean $\pm$S.E. for $n = 6$.
*Value significantly different from the respective control, $P < 0.05$.

ken and rounded outlines. According to the TUNEL assay and DNA electrophoresis, this study has revealed that SNP can cause DNA fragmentation. Cells processing apoptosis can be detected by a flow cytometer to determine unequivocal hypodiploid DNA peaks derived from the reduced DNA content in apoptotic nuclei following PI staining [29]. The percentage of apoptotic cells in osteoblasts exposed to SNP apparently increases. This is the first demonstration that NO induces apoptosis of primary normal rat calvarial osteoblasts from various aspects of cell morphology, apoptotic cells, and DNA fragmentation. Dypbukt et al. [12] report that high concentrations of NO will cause LDH release and necrotic cell death. SNP at high concentrations has the possibility of inducing osteoblast death partially through necrotic mechanisms.

NO could directly or indirectly damage osteoblast DNA. The roles of NO in cells have been implicated to have protective, regulatory, or deleterious effects [37]. As a pathophysiological molecular effector, the chemical biology of NO can be divided into direct and indirect pathways. NO is a high energy radical and has the ability to cause DNA fragmentation by direct attack or indirect activation of serious signal transduction [3,37]. Previous studies have reported that NO induced by proinflammatory cytokines, tumor necrosis factor-α, interleukin-1β and interferon-γ, or an NO donor, S-nitroso-N-acetyl-D,L-penicillamine would lead to DNA fragmentation and cell death of a mouse clonal osteogenic cell line, MC3T3-E1 cell [11,27]. The present study, using the primary culture system of normal rat calvarial osteoblasts, has shown that administration of the bone cells with SNP causes nuclear DNA fragmentation by the analyses of apoptotic cells and the DNA ladder. Therefore, it is possible that NO at high concentration would directly or indirectly damage osteoblast nuclei and lead to DNA fragmentation.

De novo synthesis of proapoptotic Bax protein could be involved in the apoptotic mechanism of NO-induced osteoblast death. Cycloheximide, an inhibitor of protein translation, partially suppresses NO-induced osteoblast apoptosis. This means that de novo protein synthesis is involved in the mechanism of NO-induced osteoblast apoptosis. Further evidence presented here from the analyses of immunocytochemistry and Western blot reveal that SNP significantly elevates the levels of proapotic Bax protein in osteoblasts. Bax protein is an important component in the NO-induced p53 independent apoptotic mechanism because the increase of Bax
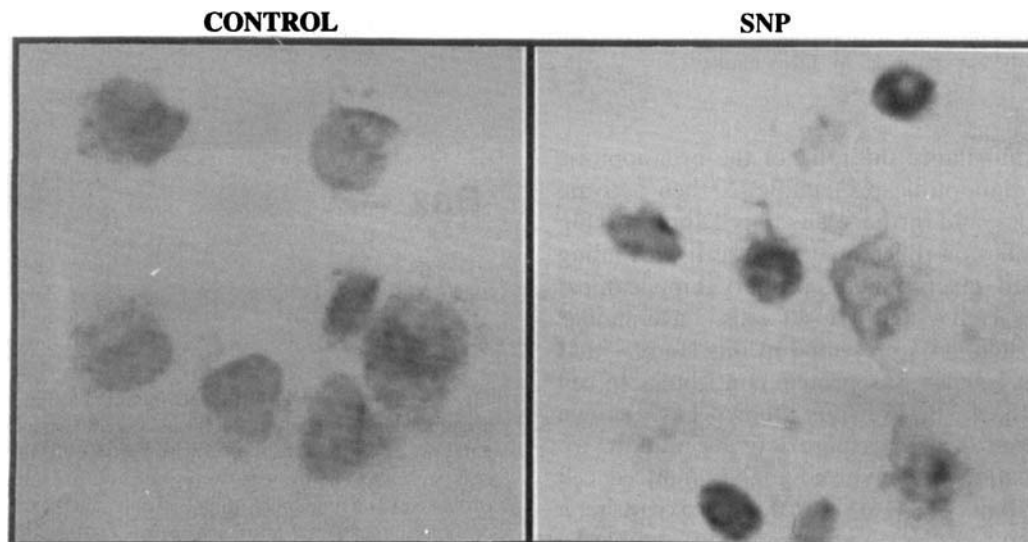

Fig. 2. SNP-induced DNA fragmentation in osteoblastic nuclei. Osteoblasts prepared from neonatal Wistar rat calvariae were treated with 2 mM SNP for 16 h. Control (C) and SNP-treated osteoblasts were harvested, and DNA fragmentation in osteoblastic nuclei were determined using the TUNEL assay as described in Materials and Methods (400×).
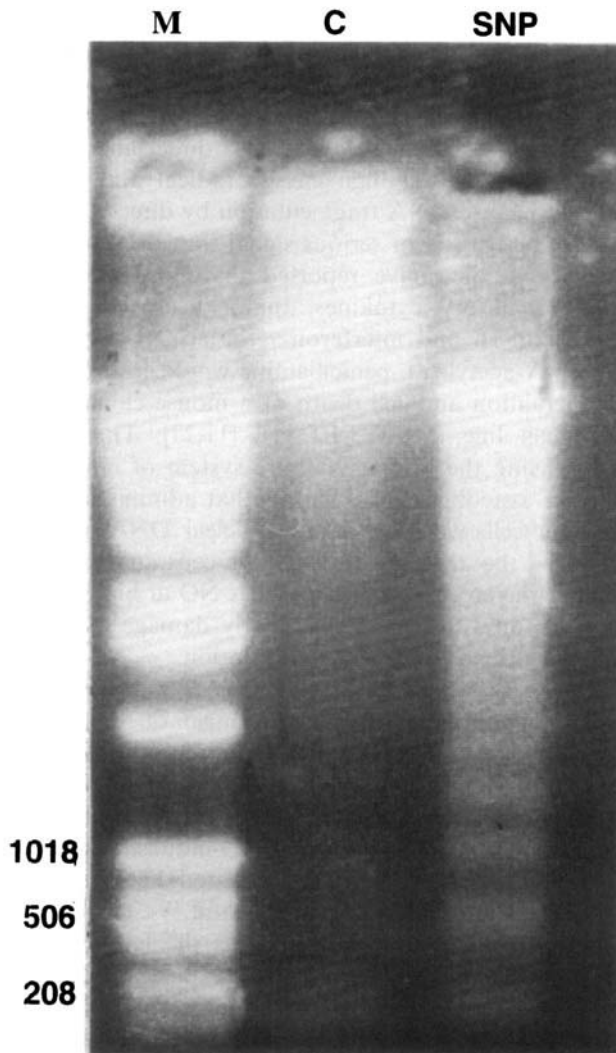
Fig. 3. SNP-induced DNA ladder in osteoblasts. Osteoblasts prepared from neonatal rat calvariae were treated with 2 mM SNP for 16 h. Genomic DNA from control (C) and SNP-treated osteoblasts were isolated and electrophoretically separated in 1.2% agarose gel containing 0.1 μg/ml ethedium bromide. M: DNA marker.
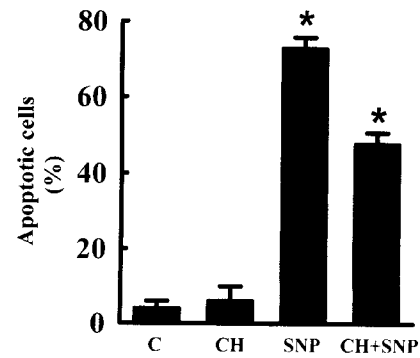

Fig. 4. Protective effects of cycloheximide (CH) on SNP-induced osteoblast apoptosis. Osteoblasts prepared from neonatal rat calvariae were pretreated with 3.6 μM CH for 1 h, and then treated with 2 mM SNP for another 16 h. Apoptotic cells from control (C), CH-, SNP- and CH and SNP-treated osteoblasts were analyzed by flow cytometry as described in Materials and Methods. Each value was represented as Mean±S.E. for $n = 9$. Value significantly different from the respective control, $P < 0.05$.
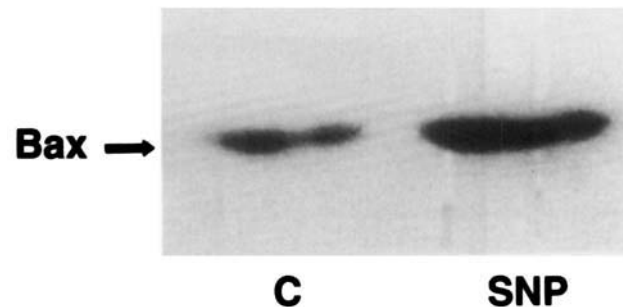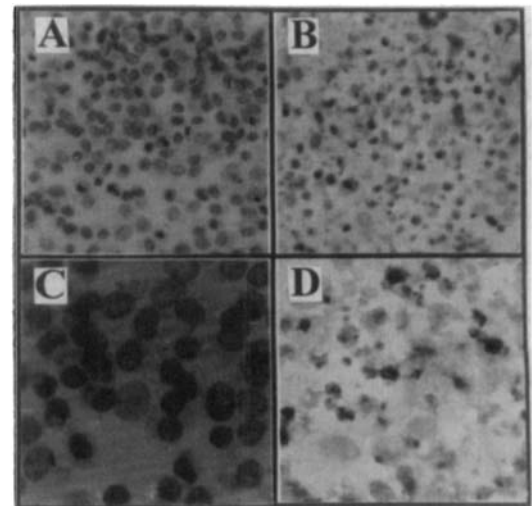

Fig. 5. Immunocytochemical and immunoblotting analyses of proapoptotic Bax protein in osteoblasts. Osteoblasts prepared from neonatal rat calvariae were treated with 2 mM SNP for 16 h. The intact cells and cytosolic proteins were prepared for immunocytochemical (top panel) and immunoblotting (bottom panel) analyses, respectively. A mouse monoclonal antibody against rat Bax protein was used in these two assays to detect Bax protein in osteoblasts. The proteins were loaded at 100 μg. Top panel: A and C – control groups, B and D – SNP-treated groups; A and B – 200×, C and D – 400×. Bottom panel: C – control.

protein would unbalance the ratio of the proapoptotic protein and antiapoptotic protein Bcl-2, then activate caspases activities, and further leads to cell apoptosis [3]. Varieties of studies on different types of cells, including T-cells, activated macrophages, primary hippocampal neurons, PC12, HelLa and HL-60 cells, have similar results as the osteoblasts presented in this study – that the elevation of cellular Bax protein contributes to cell apoptosis [17,18,24,30]. Previous studies have shown that accumulation of p53, caspase activation and cytochrome c relocation are involved in NO-induced cell apoptosis [3]. Hikiji et al. [16] show that peroxynitrite, a combined oxidant of NO and superoxide, is able to suppress ALP activity and osteocalcin expression in MC3T3-E1 cells. Our other unpublished data reveals that SNP increases intracellular reactive oxygen species

and modulates mitochondrial membrane potential. Therefore, NO itself or its oxidative metabolite such as peroxynitrite can directly induce or indirectly activate biological signaling molecules or events to cause osteoblast apoptosis.

In conclusion, the present study has used primary normal osteoblasts as an experimental model to reveal that SNP can increase the levels of NO, alternates cell morphologies, decreases cell viability and ALP activity, increases the percentage of apoptotic cells, and causes DNA fragmentation. By checking the characteristics of apoptosis, this study has demonstrated that SNP induces osteoblast death possibly through an apoptotic mechanism, and the mechanism may be involved by the de novo synthesis of proapoptotic Bax protein. To find other potential signaling effectors or pathways that mediate NO-induced osteoblast apoptosis is our next interesting subject.

Acknowledgements

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