行政院國家科學委員會補助專題研究計畫成果報告

一氧化氮誘導骨母細胞凋零研究 -探討 Caspases 所扮演之角色(1/2)

計畫類別：☒個別型計畫　□整合型計畫

計畫編號：NSC91-2314-B-038-031-

執行期間：91 年 08 月 01 日至 92 年 07 月 31 日

計畫主持人：陳瑞明

共同主持人：

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：台北醫學大學醫學系

中　華　民　國　92　年　04　月　23　日

行政院國家科學委員會專題研究計畫成果報告

一氧化氮誘導骨母細胞凋零研究-探討 Caspases 所扮演之角色 (1/2)

STUDY ON NITRIC OXIDE-INDUCED OSTEOBLAST APOPTOSIS: THE ROLE OF CASPASES

計畫編號：NSC 91-2314-B-038-031-
執行期限：91 年 08 月 01 日至 92 年 07 月 31 日
主持人：陳瑞明　台北醫學大學醫學系
計畫參與人員：林怡伶、黃景怡　台北醫學大學醫學系

Abstract

Nitric oxide contributes to osteoblast metabolism. This project is designed to determine the role of different types of caspases in the nitric oxide-induced osteoblast apoptosis using primary osteoblasts from neonatal rat calvariae as the experimental model. The first year of this project determined the role of the mitochondria-dependent caspase activation pathway, including apoptotic factors and caspase-3, in the nitric oxide-induced osteoblast apoptosis. Exposure of osteoblasts to sodium nitroprusside, a nitric oxide donor, significantly increased lactate dehydrogenase release and decreased cell viability in concentration- and time-dependent manners. Sodium nitroprusside concentration- and time-dependently caused DNA fragmentation in osteoblasts. In parallel to sodium nitroprusside-induced osteoblast apoptosis, this nitric oxide donor increased the amounts of intracellular reactive oxygen species. However, ascorbic acid and N-acetyl cystein could not block sodium nitroprusside-induced reactive oxygen species in rat osteoblasts. Administration of sodium nitruprusside significantly reduced the membrane potential and NADH oxidase activity in osteoblast mitochondria. Immunoblotting analysis revealed that sodium nitruprusside decreased the levels of Bcl-2 protein in osteoblasts. The activities of caspase-3 were time-dependently increased following sodium nitroprusside treatment. Administration of sodium nitroprusside increased the levels of 17 kD activated subunits of caspase-3. The present study has shown that nitric oxide released from sodium nitroprusside could induce osteoblast insults and apoptosis, and this might be involved by modulating intracellular oxidative stress, mitochondrial functions, anti-apoptotic Bcl-2 protein and caspase-3.

Keywords: Osteoblasts, Nitric oxide, Apoptosis, Caspase-3

Introduction

Nitric oxide (NO), one of reactive oxygen species (ROS), contributes to a variety of biological activities, including vaso-regulation, neuronal transmission, immune response, or cell apoptosis (Moncada et al., 1991; Wink et al., 1998; Brüne et al., 1999; Chang et al., 2002). For modulating bone formation and resorption, NO plays an important role in bone remodeling (Collin-Osdoby et al., 1995; Chae et al., 1997). Osteoblasts, which mediate bone formation in bone metabolism, can constitutively produce NO (Riancho et al., 1995; Collin-Osdoby et al., 1995). In response to the stimulation of pro-inflammatory cytokines, large bodies of NO will be synthesized in osteoblasts (Riancho et al., 1995; Mancini et al., 2000). NO has the biphasic effects on osteoblasts. As an effective mediator, the constitutive

expression of NO is involved in the proliferation and differentiation of human and rodent osteoblasts (O'Shaughnessy et al., 2000). As a death effector, the over-produced NO can cause osteoblast injury or even death (Armour et al., 1999; Chen et al., 2002).

Apoptosis, also named programmed cell death, is not only an evolutionarily conserved form of physiologic cell death in normal development and homeostasis but also contributes to the pathophysiology of tissue/cell injury (Goyal, 2001; Rathmell and Thompson, 2002). When associated with proliferation and differentiation, apoptosis determines the size of osteoblast population and is involved in bone remodeling in the postanal and adult skeleton (Hock et al., 2001). NO is a critical bioregulator to induce cell apoptosis (Chung et al., 2001; Chang et al., 2002). The over production of NO in osteoblasts responsive to pro-inflammatory cytokine stimulation would lead to osteoblast death (Damoulis and Hauschka, 1999; Mogi et al., 1999). Administration of NO donors such as sodium nitroprusside (SNP) would release massive levels of NO and induce osteoblast apoptosis (Messmer et al., 1996; Chen et al., 2002).

There are multiple mechanisms involved in cell apoptosis. An increase in the levels of reactive oxygen species can enhance oxidative stress and then induce cell insults (Shackelford et al., 2000). Administration of NO donors and peroxynitrite, an oxidative metabolite of NO and superoxide, would enhance cellular oxidative stress and promote cell apoptosis (Wei et al., 2000; Chung et al., 2001; Del Carlo and Loeser, 2002). Mitochondria are critical for the maintenance of cell functions. The disruption of mitochondrial activity would lead to cell dysfunction or even death (Yu et al., 2002). NO has been reported to interfere with mitochondrial functions through modulating the membrane potential and to promote the release of apoptotic factors, including, including reactive oxygen species and cytochrome c, from this organelle to the cytoplasm (Hortelano et al., 1999; Umansky et al., 2000; Pearce et al., 2001). Bcl-2 is an anti-apoptotic protein, the decrease in cellular levels would drive cells undergoing apoptosis (Srivastava et al., 1999; Brüne et al., 1999; Cheng et al., 2001). Caspases are constitutively present in cells as nonactive zymogens and require proteolytic cleavage into the catalytic active heterodimeter. Initiator or signaling caspases are activated by an upstream signal transducing step thus processing downstream or effector/executor caspases that in turn are implicated in the degradation of multiple substrates (Cohen, 1997). However, the roles of cellular oxidative stress, mitochondrial functions, Bcl-2 and caspase-3 in NO-induced osteoblast apoptosis are still unknown. This study is aimed to investigate if the NO-induced osteoblast apoptosis is involved by intracellular reactive oxygen species, mitochondrial activities, Bcl-2 protein and caspase-3 using neonatal rat calvarial osteoblasts as the experimental model.

Materials and Methods

Preparation of rat osteoblasts

Primary osteoblasts were prepared from 3-day-old Wistar rat calvaria according to the method of Partridge et al. (1981). Osteoblasts were seeded in Dulbecco's modified Eagle medium (Gibco, BRL, Grand Island, NY, USA) supplemented with 10 % heat-inactivated fetal bovine serum, L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml) in 75-cm^2 flasks at 37 °C in a humidified atmosphere of 5 % CO_2. Osteoblasts were grown to a confluence prior to the drug treatment. Only the first passage of rat osteoblasts was used in the present study.

Drug treatment

Sodium nitroprusside (SNP) purchased from Sigma Corporation (St. Louis, MO, USA) was freshly dissolved in phosphate-based saline (PBS) buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and protected from light. The concentration- and time-dependent effects of SNP on osteoblasts were determined first.

Quantification of lactate dehydrogenase

The amounts of lactate dehydrogenase released in the culture medium by osteoblasts were determined to evaluate the cytotoxicity of SNP to these cells. Osteoblasts (1×10^5) were seeded in 24-well tissue culture plates (Corning Costar Corporation, Cambridge, MA, USA). After administration of SNP, the culture medium was collected and centrifuged. The amounts of lactate dehydrogenase in supernatants were analyzed using a model 7450 automatic autoanalyzer system of Hitachi Ltd., Tokyo, Japan.

Assay of the membrane integrity

The integrity of osteoblast membrane was determined by the trypan blue exclusion method to evaluate the cytotoxicity of SNP. Briefly, osteoblasts (2×10^5) were cultured in 24-well tissue culture plates. After SNP administration, osteoblasts were trypsinized using 0.1 % trypsine-EDTA (Gibco, BRL, Grand Island, NY, USA) and harvested in 1 x PBS buffer. Following centrifuging and washing, osteoblasts were suspended in 1 x PBS buffer and stained with equal volume of trypan blue dye. The fractions of dead cells with blue color in osteoblasts were counted in a reverse-phase microscope.

Analysis of cellular DNA fragmentation

DNA fragmentation in osteoblasts were quantified by detecting BrdU-labeled histone-associated DNA fragments in the cytoplasm of cell lysates according to the instruction of the cellular DNA fragmentation ELISA kit (Boehringer Mannheim, Indianapolis, IN, USA). Briefly, osteoblasts (2×10^5) were sub-cultured in 24-well tissue culture plates and labeled with BrdU for over night. The cells were harvested and suspended in the culture medium. One hundred micrometer of cell suspension was added in each well of 96-well tissue culture plates. The cells were co-cultured with SNP for another 8 hours at 37 °C in a humidified atmosphere of 5 % CO_2. The amounts of BrdU-labeled DNA in the cytoplasm were quantified by an Anthos 2010 microplate photometer (Anthos Labtec Instruments GmbH, Lagerhausstrasse, Wals/Salzburg, Aus) at a wavelength of 450 nm.

Determination of intracellular reactive oxygen species

The levels of intracellular reactive oxygen species were quantified following the method as described previously (Liu et al., 2001). Briefly, 5×10^5 osteoblasts were cultured in 12-well tissue culture plates for overnight, and then co-treated with SNP and 2',7'-dichlorofluorescin diacetate, an reactive oxygen species sensitive dye. After drug treatment, osteoblasts were harvested and suspended in 1 x PBS buffer. The relative fluorescence intensity in osteoblasts was quantified by a flow cytometer (FACS Calibur, Becton Dickinson, San Joes, CA, USA).

Quantification of mitochondrial membrane potential

The membrane potential of mitochondria in osteoblasts was determined according to the method of Chen (1988). Briefly, osteoblasts (5×10^5) were seeded in 12-well tissue culture plates for overnight and then treated with SNP for different time intervals. After administration of SNP, osteoblasts were harvested and incubated with 3,3'-dihexyloxacarbocyanine ($DiOC_6(3)$), a positively charged dye, at 37 °C for 30 min in a humidified atmosphere of 5 % CO_2. After washing and centrifuging, the cell pellets were suspended with 1 x PBS buffer. The fluorescent intensities in osteoblasts were analyzed by a flow cytometer (FACS Calibur, Becton Dickinson, San Joes, CA, USA).

Assay of NADH-dependent dehydrogenase activity

The activity of NADH-dependent dehydrogenase were determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay following the method of Wu et al. (2003) to evaluate the effect of SNP on mitochondria. Briefly, ten thousand macrophages were seeded in 96-well tissue culture clusters for overnight. After drug treatment, cells were cultured with a new medium containing 0.5 mg/ml 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for another three hours. The blue formazan product in cells was dissolved in dimethyl sulfoxide and measured spectrophotometrically at a wavelength of 550 nm.

Caspase-3 fluorometric assay

Caspase-3 activity was determined by fluorometric reaction according to the standard protocol in the caspase-3 fluorometric assay kit (R&D Systems, Inc., Minneapolis, MN, USA). After SNP treatment, the cytosolic extracts of osteoblasts were prepared by lysing the cells in a buffer containing 1 % Nonidet P-40, 200 mM NaCl,20 mMTris/HCl, pH 7.4, 10 μg/ml leupeptin, 0.27 U/ml aprotinin and 100 μM PMSF. The cell lysate (100 μg total protein) was incubated at 37 °C for 3 hours with 50 μM the fluorogenic substrate, DEVD-AFC, in 200 μl cell-free system buffer (10 mM Hepes, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$ 5 mM pyruvate, 0.1 mM PMSF, and 1mM dithiothreitol). The release of fluorescent 7-amino-4-trifluoromethyl coumarin was measured by LS55 Luminescence Spectrometer (Perkin Elmer Instrutments, Shelton, CT, USA).

Gel electrophoresis and immunoblotting analysis

After SNP treatment, osteoblasts were washed with 1 x PBS buffer, and the cell lysates were prepared in an ice-cold radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl pH 7.2, 0.1 % SDS, 1 % Triton X-100, 1 % sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA). To avoid protein degradation, the RIPA buffer contained a mixture of proteinase inhibitors, including 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate and 5 μg/ml leupeptin. Protein concentrations were quantified by a bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Cytosolic proteins (100 μg per well) were subjected to Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to nitrocellulose membrane as described elsewhere (Chen et al., 2000). The membranes were blocked with 5 % non-fat milk at 37 °C for 1 hour. Immunodetection of Bcl-2 and caspase-3 was carried out using mouse monoclonal antibody against rat Bcl-2 and caspase-3 (Transduction Laboratories, Lexington, KY, USA). Cellular β-actin protein was immunodetected by a mouse monoclonal antibody against mouse β–actin (Sigma, Saint Louis, MI, USA) as an internal standard. Intensities of the immunoreactive bands were determined using an UVIDOCMW Version 99.03 digital imaging system (Uvtec Limited, Cambridge, England, UK).

Statistical analysis

The statistical significance of the difference between control and drug-treated groups was evaluated by the Student's *t*-test. A *P*-value less than 0.05 was considered as statistically significant. The statistical difference between groups was considered significant when the *P* value of the Duncan's multiple rang test was less than 0.05.

Results

Cytotoxicity of SNP to osteoblasts was assayed by analyzing the release of lactate dehydrogenase and the membrane integrity (Tables 1 and 2). Exposures of osteoblasts to 1.5 and 2 mM SNP for 16 hours resulted in 85 % and 2.5-fold increases of lactate dehydrogenase in the culture medium, respectively (Table 1). SNP at 0.5 and 1 mM did not affect the release of lactate dehydrogenase. Analysis of trypan blue exclusion revealed that administration of osteoblasts with 1.5 and 2 mM SNP for 16 hours decreased 38 % and 62 % cell viability, respectively (Table 1). Exposure of osteoblasts to 0.5 and 1 mM SNP did not influence cell viability.

Administration of 2 mM SNP for 8 and 16 hours significantly increased 65 % and 2.5-fold the release of lactate dehydrogenase from osteoblasts to the culture medium, respectively (Table 2). The level of lactate

dehydrogenase in culture medium was not affected by SNP treatment for 4 hours. Exposure of osteoblasts to 2 mM SNP for 8 and 16 hours significantly reduced 40 % and 55 % cell viability, respectively (Table 2). After administration of SNP for 4 hours, the cell viability was not changed.

DNA damage was quantified to determine if osteoblastss underwent apoptosis following SNP treatment. Exposure of osteoblasts to 1.5 and 2 mM SNP for 16 hours significantly increased 92 % and 3.5-fold DNA fragmentation (Fig. 1). SNP at 0.5 and 1 mM did not cause DNA damage. When exposed to 2 mM SNP for 8 and 16 hours, the ratio of DNA fragmentation in osteoblasts was significantly augmented by 65 % and 3.5-fold. Exposure of osteoblasts to 2 mM SNP for 4 hours did not lead to DNA fragmentation.

After SNP treatment, the levels of intracellular reactive oxygen species were analyzed to determine if SNP induced intracellular reactive oxygen species in osteoblasts (Fig. 2). Exposure of osteoblasts to SNP for 1 hour significantly increased 76 % intracellular reactive oxygen species (Fig. 2). After administration of SNP for 2 and 4 hours, this NO donor resulted in 3.2- and 5.3-fold increases in the levels of intracellular reactive oxygen species.

Ascorbic acid and N-acetyl cystein, two typical antioxidants, were used to block SNP-induced intracellular reactive oxygen species (Table 3). Exposure of osteoblasts to SNP significantly increased about 5-fold intracellular reactive oxygen species. The basal levels of intracellular reactive oxygen species in osteoblasts exposed to ascorbic acid and N-acetyl cystein were decreased by 54 % and 63 %, respectively. However, combined treatment of SNP with ascorbic acid or N-acetyl cystein could not abolish SNP-induced intracellular reactive oxygen species (Table 3).

The membrane potential and activity of NADH-dependent dehydrogenase were determined to evaluate the effect of SNP on mitochondrial function (Fig 3). Exposure of osteoblasts to SNP for 1 hour did not affect the membrane potential of mitochondria (Fig. 3). When the administered time intervals achieved 2 and 4 hours, SNP significantly resulted in 16 % and 36 % decreases in the membrane potential of osteoblast mitochondria. The activities of NADH-dependent dehydrogenase in osteoblast mitochondria exposed to 2 mM SNP for 8 and 16 hours were decreased by 18 % and 58 %, respectively. However, administration of osteoblasts with 2 mM SNP for 4 hours did not affect NADH-dependent dehydrogenase activity.

Bcl-2 protein in osteoblasts was immunodetected and quantified to determine the effect of SNP on theis anti-apoptotic protein (Fig 4). In untreated osteoblasts, Bcl-2 protein was detectable (Fig. 4A, *top panel*, lane 1). After SNP administration, the levels of Bcl-2 protein were significantly decreased (lane 2). The expression of β-actin was used as the internal standard (Fig. 4A, *bottom panel).* Intensities of the immunoreactive protein bands were analyzed using a digital imaging system (Fig. 4B). SNP significantly reduced 82 % Bcl-2 protein levels in osteoblasts.

Following the increase in intracellular calcium concentration, administration of osteoblasts with SNP for 1, 3, 6 and 12 hours caused significant increases in caspase-3 activity and protein, respectively (Figs. 5 and 6).

References

Armour KE, Van'T Hof RJ, Grabowski PS, Reid DM, Ralston SH: Evidence for a pathogenic role of nitric oxide in inflammation-induced osteoporosis. J Bone Min Res 14:2137-2142, 1999

Bates JN, Baker MT, Guerra Jr R, Harrison DG: Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss are required. Biochem Pharmacol 42: 157-65, 1991

Brüne B, von Knethen A, Sandau KB: Nitric oxide (NO): an effector of apoptosis. Cell Death and Differen 6: 969-975, 1999

Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB: Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47: 936-42, 1987

Chae HJ, Park RK, Chung HT, Kang JS, Kim MS, Choi DY, Bang BG, Kim HR: Nitric oxide is a regulator of bone remodelling. J Pharm Pharmacol 49:897-902, 1997

Chow JWM, Fox SW, Lean JM, Chambers TJ: Role of nitric oxide and prostaglandins in mechanically induced bone formation. J Bone Min Res 13: 1039-1044, 1998

Collin-Osdoby P, Nickols GA, Osdoby P: Bone cell function, regulation, and communication: a role for nitric oxide. J Cell Biochem 57(3):399-408, 1995

Damoulis PD, Hauschka PV: Cytokines induce nitric oxide production in mouse osteoblasts. Biochem Biophys Res Comm 201(2):924-931, 1994

Damoulis PD, Hauschka PV: Nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. J Bone Min Res 12:412-422, 1997

Dmoulis PD, Hauschka PV: Nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. J Bone Min Res 12(3):412-422, 1997

Dypbukt JM, Ankarcrona M, Burkitt M, Sjöholm Ä, Ström K, Orrenius S, Nicotera P: Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells: the role of intracellular polyamines. J Biol Chem 269: 30553-60, 1994

Evans DM, Ralston SH: Nitric oxide and bone. J Bone Min Res 11:300-305, 1996

Hara F, Fukuda K, Ueno M, Hamanishi C, Tanaka S: Pertussis toxin-sensitive G proteins as mediators of stretch-induced decrease in nitric-oxide release of osteoblast-like cells. J Ortho Res 17:593-597, 1999

Heflich MH, Evans DE, Grabowski PS, Pollock JS, Ohshima H, Ralston SH: Expression of nitric oxide synthase isoforms in bone and bone cell cultures. J Bone Min Res 12: 1108-1115, 1997

Hikiji H, Shin WS, Oida S, Takato T, Koizumi T, Toyo-oka T: Direct action of nitric oxide on osteoblastic differentiation. FEBS Letters 410:238-42, 1997

Hughes FJ, Buttery LD, Hukkanen MV, O'Donnell A, Maclouf J, Polak JM: Cytokine-induced prostaglandin E2 synthesis and cyclooxygenase-2 activity are regulated both by a nitric oxide-dependent and -independent mechanism in rat osteoblasts in vitro. J Bio Chem 274:1776-1782, 1999

Jilka RL: Cytokines, bone remodeling, and estrogen deficiency: a 1998 update. Bone 23(2):75-81, 1998

Johnson DL, McAllister TN, Frangos JA: Fluid flow stimulates rapid and continuous release of nitric oxide in osteoblasts. Am J Physiol 271:E205-8, 1996

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

Kowaluk EA, Seth P, Fung HL: Metabolic activation of sodium nitroprusside to nitric oxide in vascular smooth muscle. J Pharm Exp Ther 262, 916-922, 1992

Lamas S, Marsden PA, Li GK, Tempst P, Michel T: Endothetial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc Natl Acad Sci USA 89: 6348-6352, 1992

Lowik CW, Nibbering PH, van de Ruit M, Papapoulos SE: 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 93:1465-72, 1994

Messmer UK, Brüen B: Nitric oxide – induced apoptosis: p53-dependent and p53-independent signalling pathways. Biochem J 319: 299-305, 1995

Me β mer UK, Brüne B: Nitric Oxide (NO) in Apoptotic versus necrotic RAW 264.7 macrophage cell death: the role of NO-donor exposure, NAD^+ content, and p53 accumulation. Arch Biochem Biophys 327: 1-10, 1996

McAllister TN, Frangos JA: Steady and transient fluid shear stress stimulate NO release in osteoblasts through distinct biochemical pathways. J Bone Min Res 14(6):930-936, 1999

Moncada S, Palmer RMJ, Higgs EA: Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 43: 109-142, 1991

Moncada S, Higgs A: L-arginine-nitric oxide pathway. N Engl J Med 329: 2002-2012, 1993

Mogi M, Kinpara K, Kondo A, Togari A: Involvement of nitric oxide and biopterin in proinflammatory cytokine-induced apoptotic cell death in mouse osteoblastic cell line MC3T3-E1. Biochem Pharmacol 58(4):649-654, 1999

Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C: A rapid and simple method of measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139, 271-279, 1991

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

Parafitt AM: Bone remodelling and bone loss: understanding the physiology of osteoporosis. Clin Ostet Gynecol 30: 789-811, 1987

Partidge NC, Alcorn D, Michelangeli VP, Kemp BE, Ryan GB, Martin TJ: Functional properties of hormonally responsive cultured normal and malignant rat osteoblastic cells. Endocrinology 108: 213-219, 1981

Pitsillides AA, Rawlinson SC, Suswillo RF, Bourrin S, Zaman G, Lanyon LE: Mechanical strain-induced NO production by bone cells: a possible role in adaptive bone (re)modeling?. FASEB Journal. 9(15):1614-1622, 1995

Ralston SH, Todd D, Helfrich M, Benjamin N, Grabowski PS: Human osteoblast-like cells produce nitric oxide and express inducible nitric oxide synthase. Endocrinology. 135:330-336, 1994

Riancho JA, Salas E, Zarrabeitia MT, Olmos JM, Amado JA, Fernandez-Luna JL, Gonzalez-Macias J: Expression and functional role of nitric oxide synthase in osteoblast-like cells. J Bone Min Res 10(3):439-446, 1995

Riancho JA, Zarrabeitia MT, Fernandez-Luna JL, Gonzalez-Macias J: Mechanisms controlling nitric oxide synthesis in osteoblasts. Mol Cell Endocrinol 107(1):87-92, 1995

Shen YH, Wang XL, Wilcken DEL: Nitric oxide induces and inhibits apoptosis through different pathways. FEBS Lett 433: 125-131, 1998

Smalt R, Mitchell FT, Howard RL, Chambers TJ: Induction of NO and prostaglandin E2 in osteoblasts by wall-shear stress but not mechanical strain. Am J Physiol 273:E751-E758, 1997

Tam VK, Schotland S, Green J: Inflammatory cytokines (IL-1alpha, TNF-alpha) and LPS modulate the Ca2+ signaling pathway in osteoblasts. Am J Physiol 274:C1686-C1698, 1998

Ueno M, Fukuda K, Oh M, Asada S, Nishizaka F, Hara F, Tanaka S: Protein kinase C modulates the synthesis of nitric oxide by osteoblasts. Cal Tissue Intl. 63:22-26, 1998

Wink DA, Mitchell JB: Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Rad Biol Med 25: 434-456, 1998

Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan C: Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 1992; 256: 225-8.

Zaman G, Pitsillides AA, Rawlinson SC, Suswillo RF, Mosley JR, Cheng MZ, Platts LA, Hukkanen M, Polak JM,

Lanyon LE: Mechanical strain stimulates nitric oxide production by rapid activation of endothelial nitric oxide synthase in osteocytes. J Bone Min Res 14:1123-1131, 1999

Table 1 Concentration-dependent effects on the cytotoxicity of sodium nitroprusside to rat osteoblasts

SNP, mM	Lactate dehydrogenase (U/L)	Cell viability (cell number x 10^3)
0	41 ± 11	188 ± 44
0.5	38 ± 10	187 ± 38
1	35 ± 8	179 ± 45
1.5	76 ± 8*	116 ± 18*
2	104 ± 13*	72 ± 21*

Rat osteoblasts were exposed to 0.5, 1, 1.5 and 2 mM sodium nitroprusside (SNP) for 16 hours. The amounts of lactate dehydrogenase released from osteoblasts to culture medium were analyzed by an autoanalyzer as described in Materials and method. Cell viability was determined by the trypan blue exclusion method. Each value was expressed as Mean ± SEM for n = 12. *Values were considered to have statistical difference from the respective control, $P < 0.05$.

Table 2 Time-dependent effects on the cytotoxicity of sodium nitroprusside to rat osteoblasts

Time, h	Lactate dehydrogenase (U/L)	Cell viability (cell number x 10^3)
0	37 ± 10	179 ± 31
4	30 ± 6	167 ± 58
8	61 ± 9*	107 ± 30*
16	93 ± 11*	81 ± 19*

Rat osteoblasts were exposed to 2 mM sodium nitroprusside for 0, 4, 8 and 16 hours. The amounts of lactate dehydrogenase released from osteoblasts to culture medium were analyzed by an autoanalyzer as described in Materials and method. Cell viability was determined by the trypan blue exclusion method. Each value was expressed as Mean ± SEM for n = 12. *Values were considered to have statistical difference from the respective control, $P < 0.05$.

Table 3 Effects of ascorbic acid and N-acetyl cysteine on sodium nitroprusside-induced intracellular reactive oxygen species in rat osteoblasts

	iROS, folds of control
Control	1
SNP	4.68 ± 0.36*
AA	0.46 ± 0.02*
AA + SNP	5.83 ± 0.77*
NAC	0.37 ± 0.03*
NAC + SNP	6.13 ± 1.02*

Rat osteoblasts were exposed to 2 mM sodium nitroprusside (SNP), 30 μM ascorbic acid (AA), 1 mM N-acetyl cysteine (NAC), a combination of AA and SNP and a combination of NAC and SNP for 4 hours. The amounts of intracellular reactive oxygen species (iROS) were quantified by the flow cytometric method. Each value was expressed as Mean ± SEM for n = 6. *Values were considered to have statistical difference from the respective control, $P < 0.05$.

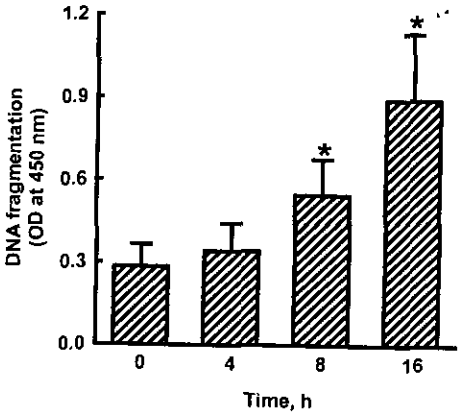

Fig. 1 Time-dependent effects of SNP on DNA fragmentation of rat osteoblasts.

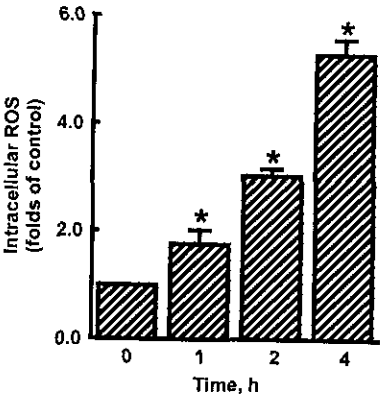

Fig. 2 Time-dependent effects of SNP on intracellular reactive oxygen species of rat osteoblasts.

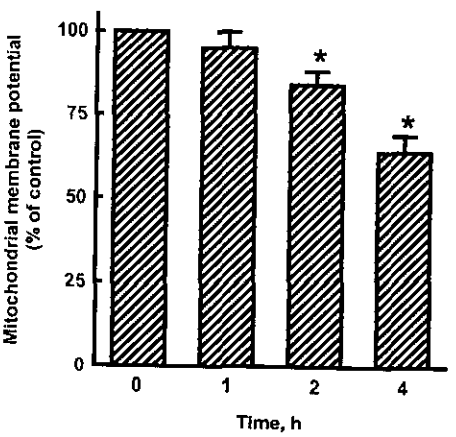

Fig. 3 Time-dependent effects of SNP on mitochondrial membrane potential of rat osteoblasts.

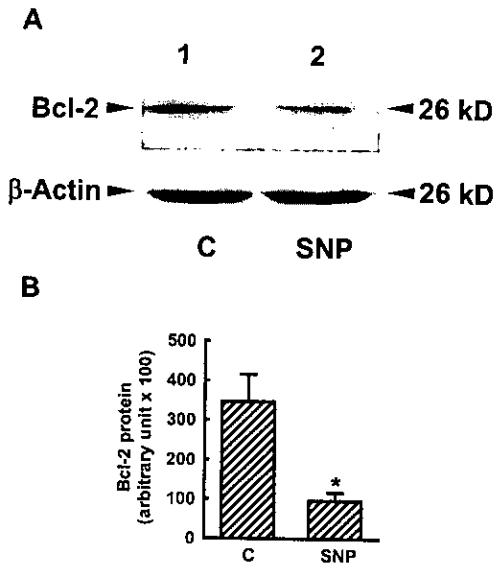

Fig. 4 Effects of SNP on Bcl-2 protein of rat osteoblasts.

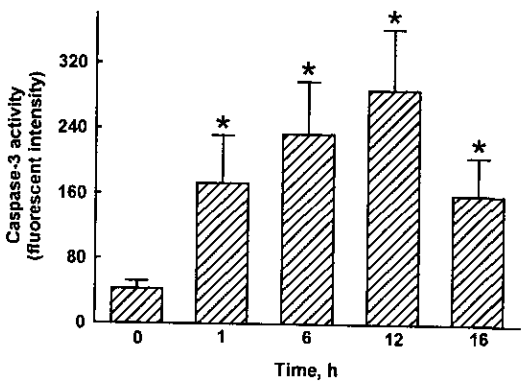

Fig. 5 Efffects of SNP on caspase-3 activity of rat osteoblasts.

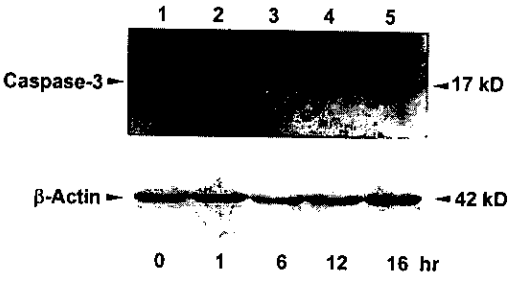

Fig. 6 Effects of SNP on the activated subunit of caspase-3 protein.