Nitric Oxide Protects Osteoblasts from Oxidative Stress-Induced Apoptotic Insults via a Mitochondria-Dependent Mechanism

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ABSTRACT: Nitric oxide (NO) contributes to the regulation of osteoblast activities. In this study, we evaluated the protective effects of NO pretreatment on oxidative stress-induced osteoblast apoptosis and its possible mechanism using neonatal rat calvarial osteoblasts as the experimental model. Exposure of osteoblasts to sodium nitroprusside (SNP) at a low concentration of 0.3 mM significantly increased cellular NO levels without affecting cell viability. However, when the concentration reached a high concentration of 2 mM, SNP increased the levels of intracellular reactive oxygen species and induced osteoblast injuries. Thus, administration of 0.3 and 2 mM SNP in osteoblasts were respectively used as sources of NO and oxidative stress. Pretreatment with NO for 24 h significantly ameliorated the oxidative stress-caused morphological alterations and decreases in alkaline phosphatase activity, and reduced cell death. Oxidative stress induced osteoblast death via an apoptotic mechanism, but NO pretreatment protected osteoblasts against the toxic effects. The mitochondrial membrane potential was significantly reduced following exposure to the oxidative stress. However, pretreatment with NO significantly lowered the suppressive effects. Oxidative stress increased cellular Bax protein production and cytochrome *c* release from mitochondria. Pretreatment with NO significantly decreased oxidative stress-caused augmentation of Bax and cytochrome *c* protein levels. In parallel with cytochrome *c* release, oxidative stress induced caspase-3 activation and DNA fragmentation. Pretreatment with NO significantly reduced the oxidative stress-enhanced caspase-3 activation and DNA damage. Results of this study show that NO pretreatment can protect osteoblasts from oxidative stress-induced apoptotic insults. The protective action involves a mitochondria-dependent mechanism. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:1917–1925, 2006

Keywords: osteoblasts; nitric oxide; oxidative stress; antiapoptosis; mitochondria-dependent mechanism

INTRODUCTION

Osteoblast-mediated bone formation is a critical process in bone remodeling.[1] A balance among proliferation, differentiation, and apoptosis determines the population size of osteoblasts in the skeleton.[2] A variety of systemic and local factors are involved in the regulation of osteoblast activities.[3] Nitric oxide (NO), synthesized from L-arginine by NO synthases, has been shown to be one such factor.[4] Osteoblasts can produce NO constitutively.[5] Under stimulation of inflammatory cytokines or mechanical stress, overproduction of NO by osteoblasts occurs.[6,7] NO has biphasic effects on osteoblast metabolism.[8] Overproduction of NO can lead to osteoblast injuries.[7,9,10] However, constitutive NO can be an effective mediator that regulates osteoblast proliferation and differentiation.[11]

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Apoptosis, energy-dependent cell death, plays an important role in physiological and pathophysiological regulation of tissue homeostasis and cell activities.[12] A previous study showed that apoptosis determines the osteoblast population and bone turnover in the adult skeleton.[2] Maintenance of mitochondrial functions is critical in preventing apoptotic insults from occurring to cells. Increases in the synthesis or translocation of the proapoptotic Bax protein can trigger depolarization of the mitochondrial membrane potential, enhancing the release of apoptotic factors such as cytochrome *c*, and ultimately leading to cell apoptosis.[13–15] Being an effector for death regulation, NO has been shown to decrease bone mineral density through induction of osteoblast apoptosis in inflammation-induced osteoporosis.[16] Our previous studies showed that NO can activate mitochondrial apoptotic signals that induce osteoblast apoptosis.[6,9]

NO has protective effects on stress-induced cellular insults. In 6-hydroxydopamine-induced injuries of PC12 cells, NO can activate PKG/PI3K/Akt-dependent Bad phosphorylation and prevent cells from undergoing apoptosis.[17] Serum deprivation causes apoptotic cortical neuron death, but pretreatment with low levels of the NO donor, S-nitroso-N-acetylpenicillamine, significantly reduced such toxic effects.[18,19] In ischemia and reperfusion injury, NO preconditioning was shown to produce protective effects on cardiomyocytes, hepatocytes, and endothelial cells.[20] It was suggested that NO is a mediator of estrogen action on bone in ovariectomized rats.[21] In this study, we used neonatal rat calvarial osteoblasts as the experimental model to evaluate the effects of the NO pretreatment on oxidative stress-induced cellular insults and the possible mechanisms.

MATERIALS AND METHODS

Osteoblast Isolation and Drug Treatment

Rat osteoblasts were prepared from 3-day-old Wistar rat calvaria according to the method of Partridge et al.[22] Osteoblasts were seeded in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) 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 confluence prior to drug treatment. Only the first passage of rat osteoblasts was used in the present study.

Sodium nitroprusside (SNP) purchased from Sigma (St. Louis, MO) was freshly dissolved in phosphate-based saline (PBS) buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) and protected from light. Our preliminary study revealed that administration of a low concentration of SNP (0.3 mM) in osteoblasts significantly increased the levels of cellular NO without affecting cell viability. However, SNP at a high concentration (2 mM) caused significant increases in the levels of intracellular reactive oxygen species (ROS) and induced cell injuries. Thus, SNP at 0.3 and 2 mM were used in this study as the sources of NO and oxidative stress, respectively.

Determination of Cellular Nitric Oxide Levels

Cellular NO levels were determined according to a technical bulletin of the Bioxytech NO assay kit (OXIS International, Portland, OR). After drug administration, the culture medium was collected and centrifuged. The supernatant fractions were reacted with nitrate reductase. Following a reaction of the supernatant with sulfanilamide and *N*-1-napthylethylenediamine, a colorimetric azo compound was formed and quantified using an Anthos 2010 microplate photometer (Anthos Labtec Instruments, Lagerhausstrasse, Wals/Salzburg, Austria).

Determination of Cellular Oxidative Stress

The levels of intracellular ROS were quantified to determine the cellular oxidative stress to osteoblasts according to a previously described method.[23] Briefly, 5×10^5 osteoblasts were cultured in 12-well tissue culture plates overnight, and then cotreated with SNP and 2′,7′-dichlorofluorescin diacetate, an ROS-sensitive dye. After drug treatment, osteoblasts were harvested and suspended in 1× PBS buffer. Relative fluorescence intensities in osteoblasts were quantified using a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA).

Assay of Cell Viability

Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously.[24] Briefly, osteoblasts (2×10^4) were seeded in 96-well tissue culture plates overnight. After drug treatment, osteoblasts were cultured with new medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for a further 3 h. The blue formazan products in osteoblasts were dissolved in dimethyl sulfoxide and spectrophotometrically measured at a wavelength of 550 nm.

Assay of Alkaline Phosphatase Activity

After drug treatment, alkaline phosphatase (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 by Sigma Diagnostics Alkaline, Acid and Prostatic Acid Phosphatase kit (Sigma). The ALP results were adjusted by the total protein levels.

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Analysis of Apoptotic Cells

Apoptotic osteoblasts were determined using propidium iodide to detect DNA fragments in nuclei according to a method described previously.[25] After drug administration, osteoblasts were harvested and fixed in cold 80% ethanol. Following centrifugation and washing, fixed cells were stained with propidium iodide and analyzed using a FACScan flow cytometer (Becton Dickinson) on the basis of a 560-nm dichroic mirror and a 600-nm bandpass filter.

Quantification of the Mitochondrial Membrane Potential

The mitochondrial membrane potential was determined following the method of Chen.[26] Briefly, osteoblasts (5×10^5) were seeded in 12-well tissue culture plates overnight, and then treated with drugs. After drug administration, osteoblasts were harvested and incubated with 3,3′-dihexyloxacarbocyanine ($DiOC_6$), a positively charged dye, at 37°C for 30 min in a humidified atmosphere of 5% CO_2. After washing and centrifugation, cell pellets were suspended in 1× PBS buffer. Intracellular fluorescent intensities were analyzed using a flow cytometer (FACS Calibur).

Gel Electrophoresis and Immunoblotting Analysis

After drug treatment, osteoblasts were washed with 1× PBS buffer. Cell lysates were prepared in 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, and 1 mM EDTA). To avoid protein degradation, a mixture of proteinase inhibitors, including 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate, and 5 μg/mL leupeptin, was added to the RIPA buffer. Protein concentrations were quantified using a bicinchonic acid protein assay kit (Pierce, Rockford, IL). Cytosolic proteins (100 μg per well) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk at 37°C for 1 h. Immunodetection of Bax and cytochrome *c* was carried out using mouse monoclonal antibodies against rat Bax and human cytochrome *c* proteins, respectively (Transduction Laboratories, Lexington, KY). Cellular β-actin protein was immunodetected using a mouse monoclonal antibody against mouse β-actin (Sigma) as an internal standard. Intensities of the immunoreactive bands were determined using an UVIDOCMW version 99.03 digital imaging system (UVtec, Cambridge, UK).

Assay of Caspase-3 Activity

The activity of caspase-3 was determined by a fluorogenic substrate assay. Briefly, cell extracts were prepared by lysing osteoblasts in a buffer containing 1% Nonidet P-40, 200 mM NaCl, 20 mM Tris/HCl (pH 7.4), 10 μg/mL leupeptin, 0.27 U/mL aprotinin, and 100 μM PMSF. Caspase-3 activity was determined by incubating cell lysates (25 μg total protein) with 50 μM fluorogenic substrate IETD in 200 μL of a cell-free system buffer consisting of 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 1 mM dithiothreitol. Intensities of fluorescent products in cells were measured with a spectrofluorometer.

Quantification of DNA Fragmentation

DNA fragmentation in osteoblasts was quantified to evaluate if oxidative stress damaged the nuclear DNA. BrdU-labeled histone-associated DNA fragments in the cytoplasm of cell lysates were detected according to the instructions of the cellular DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Indianapolis, IN). Briefly, osteoblasts (2×10^5) were subcultured in 24-well tissue culture plates and labeled with BrdU overnight. Cells were harvested and suspended in the culture medium. One hundred microliters of cell suspension was added to each well of 96-well tissue culture plates. Osteoblasts were cocultured with SNP for another 8 h at 37°C in a humidified atmosphere of 5% CO_2. Amounts of BrdU-labeled DNA in the cytoplasm were quantified using an Anthos 2010 microplate photometer (Anthos Labtec Instruments, Lagerhausstrasse, Wals/Salzburg, Austria) at a wavelength of 450 nm.

Statistical Analysis

Statistical differences between the control and drug-treated groups were considered significant when the *p*-value of Duncan's multiple-range test was less than 0.05. Statistical analysis between drug-treated groups was carried out using two-way ANOVA.

RESULTS

To establish the sources of NO and oxidative stress in this study, osteoblasts were exposed to SNP at low (0.3 mM) and high (2 mM) concentrations. Pretreatment of 0.3 mM SNP for 24 h significantly increased the NO levels in osteoblasts by about 30% but did not affect cell viability (data not shown). When the concentration reached 2 mM, SNP increased 6.5-fold cellular NO levels. Administration of 2 mM SNP in osteoblasts significantly augmented 13.5-fold producing intracellular ROS in osteoblasts (Table 1). In parallel with the increase in oxidative stress, the ALP activity and cell viability decreased by 80 and 88%, respectively. Therefore, exposure to low (0.3 mM) and high (2 mM) concentrations of SNP was used to produce NO and oxidative stress, respectively.

To evaluate the protective effects of NO on oxidative stress-induced osteoblast injuries, the

Table 1. Effects of Sodium Nitroprusside on Cellular Oxidative Stress, Alkaline Phosphatase Activity, and Cell Viability

Treatment	Cellular Oxidative Stress (Multiples of Control)	ALP Activity (OD Value at 410 nm)	Cell Viability (OD Value at 550 nm)
Control	1	0.89 ± 0.19	1.07 ± 0.26
SNP	13.5 ± 2.3*	0.18 ± 0.05*	0.13 ± 0.03*

Rat osteoblasts were exposed to 2 mM sodium nitroprusside (SNP) for 24 h. Cellular oxidative stress was determined using flow cytometer. Alkaline phosphatase (ALP) activity and cell viability were assayed by colorimetric methods. Each value represents the mean ± SEM for $n = 12$.

*Values significantly differ from the respective control, $p < 0.05$.

cell morphologies, ALP activity, and cell viability were analyzed (Fig. 1). Exposure to oxidative stress altered the morphologies of osteoblasts (Fig. 1A). Pretreatment with low levels of NO for 24 h did not influence cell morphologies. NO pretreatment for 24 h significantly alleviated oxidative stress-induced morphological alterations. Subjecting osteoblasts to oxidative stress significantly decreased ALP activity by 86% (Fig. 1B). Pretreatment with NO did not affect the enzyme activity. NO pretreatment significantly alleviated 48% of the oxidative stress-induced reduction in ALP activity. The viability of osteoblasts decreased by 89% following administration of oxidative stress (Fig. 1C). Pretreatment with NO was not cytotoxic to osteoblasts. After pretreatment with NO, the viability rebounded by 46%.

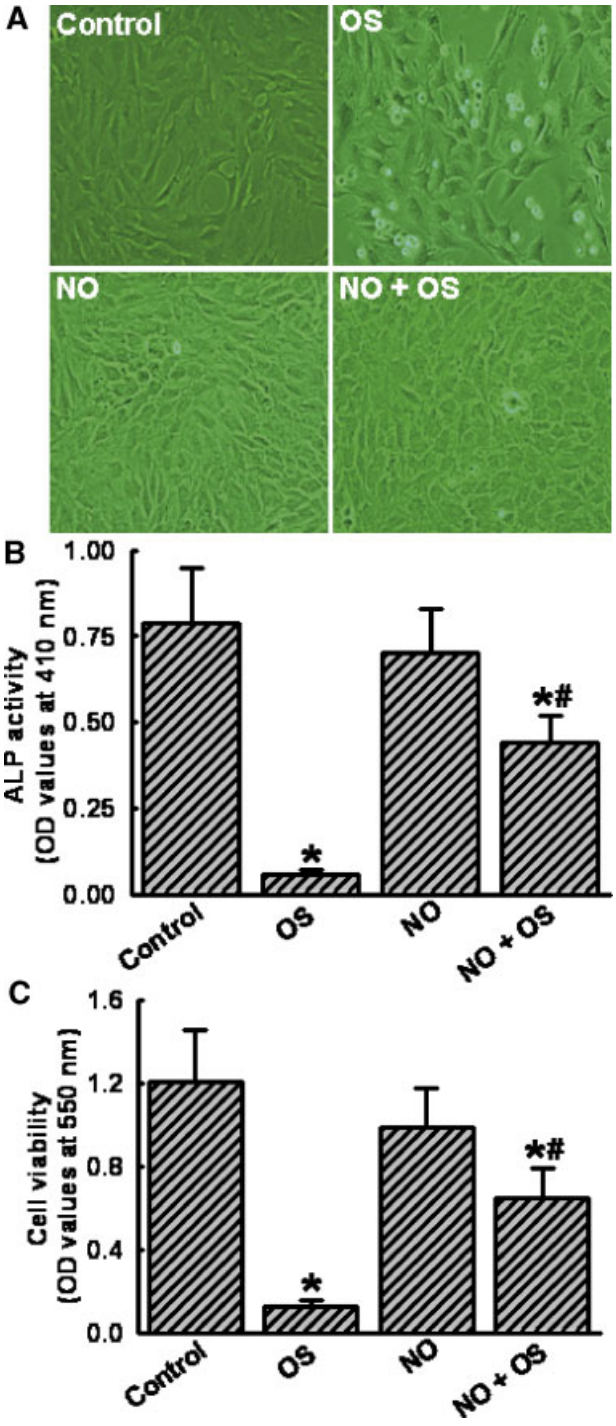
The proportion of apoptotic cells was determined to validate the antiapoptotic effects of NO on oxidative stress-caused osteoblast insults (Fig. 2). Exposure of osteoblasts to oxidative stress significantly increased the percentage of osteoblasts arrested at the sub-G1 phase (Fig. 2A). Pretreatment with NO had no action on the cell cycle. NO pretreatment significantly reduced the oxidative stress-induced sub-G1 arrest. The results were quantified and statistically analyzed (Fig. 2B). Oxidative stress caused 90% of osteoblasts to undergo apoptosis. Pretreatment with NO did not affect cell apoptosis. However, NO pretreatment significantly decreased the percentage of osteoblasts undergoing apoptosis by 60% (Fig. 2B).

To determine the role of mitochondria in NO's protection against oxidative stress, the mitochondrial membrane potential was analyzed (Fig. 3). After exposure to oxidative stress, the mitochondrial membrane potential peak shifted to the left (Fig. 3A). NO pretreatment did not cause a significant shift in the membrane potential peak. Pretreatment with NO allowed the oxidative stress-caused shift in the mitochondrial membrane potential to recover. The results were quantified and statistically analyzed (Fig. 3B). Oxidative stress caused a significant decrease of 47% in the mitochondrial membrane potential. NO pretreatment did not affect the membrane potential of osteoblast mitochondria. After pretreatment with NO, the oxidative stress-induced reduction in the mitochondrial membrane potential completely recovered (Fig. 3B).

Protein levels of Bax and cytochrome *c* were immunodetected to evaluate the mechanism of NO's protection in osteoblasts (Fig. 4). In untreated osteoblasts, Bax protein was detectable (Fig. 4A, top, lane 1). Oxidative stress increased Bax protein synthesis (lane 2). Pretreatment with NO decreased Bax protein production (lane 3). NO pretreatment significantly lowered oxidative stress-induced Bax protein synthesis (lane 4). Cytochrome *c* was detected in untreated osteoblasts (Fig. 4A, middle, lane 1). Oxidative stress augmented cytochrome *c* release from mitochondria (lane 2). Pretreatment with NO slightly increased the levels of this apoptotic protein (lane 3). However, after pretreatment with NO, the oxidative stress-induced release of cytochrome *c* decreased (lane 4). Levels of β-actin were determined as the internal control (Fig. 4A, bottom). These immunodetected protein bands were quantified and statistically analyzed (Fig. 4B). Exposure to oxidative stress significantly increased the levels of Bax and cytochrome c by 3.5- and 7.8-fold, respectively. However, pretreatment with NO significantly decreased the oxidative stress-induced syntheses of Bax and cytochrome *c* proteins (Fig. 4B).

To further verify the signal-transducing mechanism of NO's protection against oxidative stress in osteoblasts, caspase-3 activity and DNA fragmentation were determined (Fig. 5). Oxidative stress in osteoblasts activated a significant 2.8-fold increase in caspase-3 activities (Fig. 5A). Pretreatment with NO did not influence enzyme activity. After pretreatment with NO, the activity of

caspase-3 completely recovered. Oxidative stress induced 4.6-fold higher DNA fragmentation in osteoblasts (Fig. 5B). Pretreatment with NO did not cause DNA damage. NO pretreatment significantly reduced 51% of the oxidative stress-induced DNA fragmentation.



DISCUSSION

Administration of SNP at a high concentration of 2 mM caused oxidative stress to osteoblasts and induced cell damage. The levels of oxidative stress in osteoblasts were determined by 2′,7′-dichlorofluorescin diacetate, an ROS-sensitive dye. An increase in the levels of intracellular ROS means that the oxidative stress to osteoblasts was augmented. SNP can be decomposed to NO under light exposure or in the presence of a biological reducing system.[27] Because 2′,7′-dichlorofluorescin diacetate can catch peroxides and NO,[28] the sources of oxidative stress after SNP administration included NO itself and peroxynitride ($NOOO^-$), an oxidative product of NO and superoxide. In parallel with the enhancement of oxidative stress, ALP activity and cell viability decreased. Previous studies have reported that NO decomposed from SNP or other NO donors, including NOC-7 and NOC-18, induces insults to primary osteoblasts or osteoblast-like ROB-C26 and MC3T3-E1cells.[29–31] Thus, a high concentration of SNP (2 mM) caused massive oxidative stress via production of intracellular ROS, and induced osteoblast damage or even cell death.

SNP at a low concentration of 0.3 mM increased the levels of cellular NO without affecting cell viability but protected osteoblasts from oxidative stress-induced cell insults. After administration of 0.3 mM SNP for 24 h, the nitrite levels, an oxidative product of NO, were augmented by almost 30% (data not shown). The increases in nitrite levels indicated enhanced cellular NO levels. Under such conditions, the viability of osteoblasts was not affected. However, pretreatment with a low concentration of SNP for 24 h, producing low levels of NO, allowed the oxidative stress-caused suppression of ALP activity and cell viability to recover.

Figure 1. Effects of nitric oxide (NO) on oxidative stress (OS)-induced osteoblast insults. Osteoblasts prepared from neonatal rat calvaria were pretreated with a low concentration of sodium nitroprusside (0.3 mM) for 24 h as the source of NO, and then exposed to a high concentration of sodium nitroprusside (2 mM) as the source of OS, or a combination of these two treatments for another 24 h. Cell morphologies were observed with an inverse light microscope (A). Cell viability (B) and alkaline phosphatase (ALP) activity (C) were assayed using a colorimetric method. Each value represents the mean ± SEM for $n = 12$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and OS-treated groups, respectively. [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

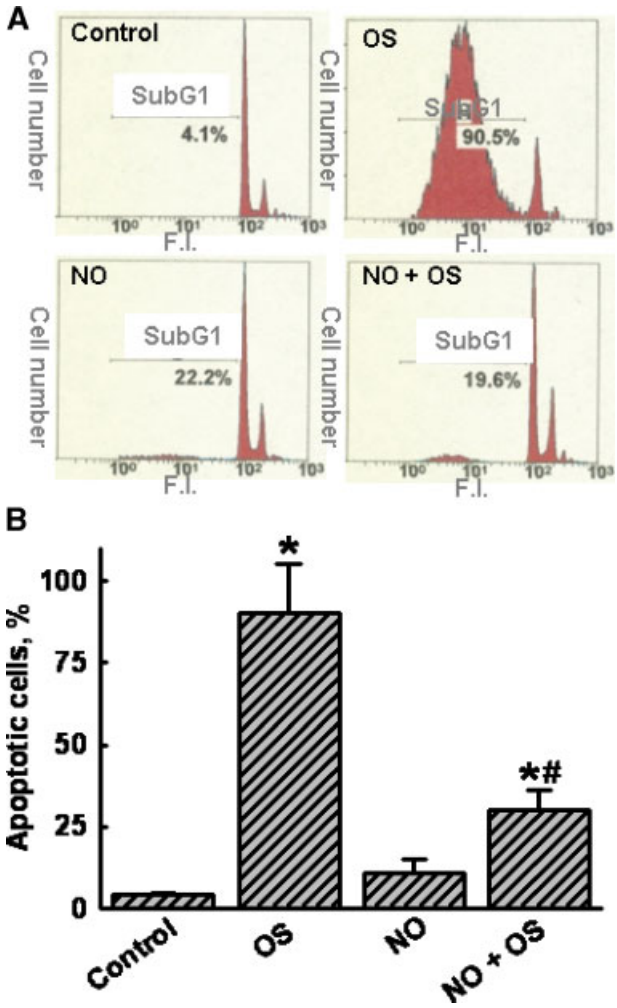


Figure 2. Antiapoptotic effects of nitric oxide (NO) on oxidative stress (OS)-induced osteoblast death. Osteoblasts prepared from neonatal rat calvaria were pretreated with a low concentration of sodium nitroprusside (0.3 mM) for 24 h as the source of NO, and then exposed to a high concentration of sodium nitroprusside (2 mM) as the source of OS, or a combination of these two treatments for another 24 h. Genomic DNA was stained with propidium iodide, and apoptotic cells were quantified using flow cytometry (A). Data were statistically analyzed as shown in (B). Each value represents the mean ± SEM for $n = 6$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and OS-treated groups, respectively. [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

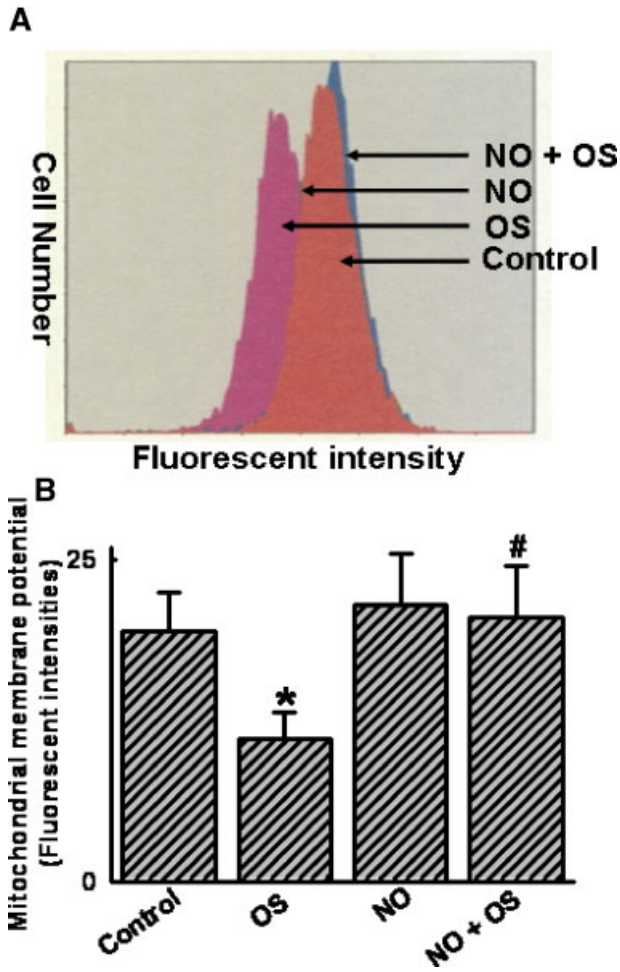


Figure 3. Effects of nitric oxide (NO) on oxidative stress (OS)-caused suppression of the mitochondrial membrane potential. Osteoblasts prepared from neonatal rat calvaria were pretreated with a low concentration of sodium nitroprusside (0.3 mM) for 24 h as the source of NO, and then exposed to a high concentration of sodium nitroprusside (2 mM) as the source of OS, or a combination of these two treatments for another 6 h. The mitochondrial membrane potential of osteoblasts was analyzed using the fluorescent dye, $DiOC_6$, and quantified by flow cytometry (A). Data were statistically analyzed as shown in (B). Each value represents the mean ± SEM for $n = 6$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and OS-treated groups, respectively. [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

In human osteosarcoma MG63 cells, pretreatment with a low concentration of SNP (0.3 mM) also significantly lowered 2 mM SNP-induced cell damage (data not shown). Our further study showed that pretreatment with a low concentration of S-nitrosoglutathione (0.5 mM), another NO donor, for 24 h significantly increased cellular NO levels and ameliorated oxidative stress-induced osteoblast insults. Thus, pretreatment with NO, decomposed from low concentrations of NO donors, has protective effects on oxidative stress-induced osteoblast insults.

The mechanism of NO's protection against oxidative stress-induced osteoblast death is via an antiapoptotic pathway. This study showed that

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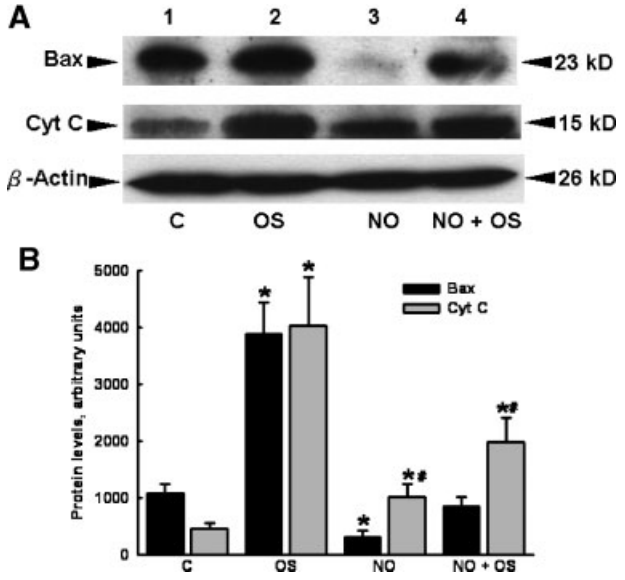

Figure 4. Effects of nitric oxide (NO) on syntheses of Bax and cytochrome *c* (Cyt *c*). Osteoblasts prepared from neonatal rat calvaria were pretreated with a low concentration of sodium nitroprusside (0.3 mM) for 24 h as the source of NO, and then exposed to a high concentration of sodium nitroprusside (2 mM) as the source of OS, or a combination of these two treatments for another 6 h. Cytosolic protein was prepared, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Immunodetection of Bax and Cyt *c* proteins was carried out using antibodies against rat Bax and human Cyt *c* proteins, respectively (A, top and middle). β-Actin was immunodetected as an internal control (bottom panel). Intensities of protein bands were quantified using a digital imaging system (B). Each value represents the mean ± SEM for $n = 6$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and OS-treated groups, respectively.

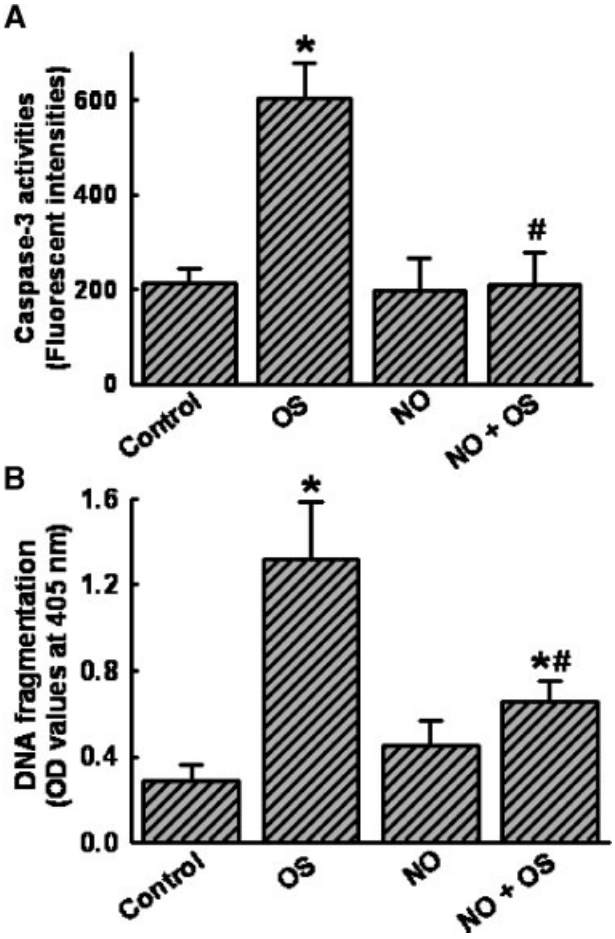

Figure 5. Effects of nitric oxide (NO) on oxidative stress (OS)-induced caspase-3 activation and DNA fragmentation. Osteoblasts prepared from neonatal rat calvaria were pretreated with a low concentration of sodium nitroprusside (0.3 mM) for 24 h as the source of NO, and then exposed to a high concentration of sodium nitroprusside (2 mM) as the source of OS, or a combination of these two treatments for another 12 h. Caspase-3 activity was determined using a fluorogenic substrate assay kit (A). DNA fragments in osteoblasts were quantified using a BrdU-labeled histone-associated DNA fragmentation ELISA kit (B). Each value represents the mean ± SEM for $n = 6$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and OS-treated groups, respectively.

oxidative stress caused shrinkage of osteoblast morphologies. Analysis of the cell cycle revealed that oxidative stress increased the percentage of osteoblasts arrested at the sub-G1 phase. The appearance of a hypodiploid sub-G1 peak represents cells undergoing apoptosis.[32] Our previous study provided several lines of evidence, including morphological alterations as well as the TUNEL assay and analysis of DNA ladders, to show that SNP-caused oxidative stress induced osteoblast apoptosis.[9] Pretreatment of NO obviously ameliorated the oxidative stress-caused osteoblast shrinkage and sub-G1 arrest. Thus, the protective mechanism of NO pretreatment is through suppression of oxidative stress-induced apoptotic damage.

Pretreatment of osteoblasts with NO significantly downregulated the oxidative stress-caused increases in cellular Bax protein levels. Oxidative stress in osteoblasts enhanced the synthesis of Bax protein. Bax is a proapoptotic protein.[15] The translocation of Bax protein from the cytoplasm to mitochondria leads to mitochondrial dysfunction and increases the risk that osteoblasts will undergo apoptosis.[33] The results provided in our previous study revealed that the de novo synthesis of Bax protein mediates NO overproduction involved in osteoblast apoptosis.[9] Thus, enhanced Bax protein

production is a critical effector triggering osteoblast apoptosis induced by oxidative stress. After pretreatment with NO for 24 h, the oxidative stress-augmented Bax protein synthesis significantly decreased. Our further unpublished data from confocal microscopic analysis revealed that pretreatment with NO obviously restored the oxidative stress-induced decreases in cellular Bcl-2 protein levels and its translocation from the cytoplasm to mitochondria. Therefore, modulation of Bax and Bcl-2 protein levels is one of important reasons explaining why NO pretreatment has antiapoptotic effects on oxidative stress-caused osteoblast death.

Pretreatment with NO significantly ameliorated the oxidative stress-caused suppression of the mitochondrial membrane potential and osteoblast apoptosis. Oxidative stress decreased the mitochondrial membrane potential of osteoblasts. Such suppression may have been due to increases in cellular Bax protein levels. Depolarization of the mitochondrial membrane potential can enhance the release of apoptotic factors from mitochondria to the cytoplasm and induce cell apoptosis.[13,14] Pretreatment of NO completely ameliorated the oxidative stress-caused decreases in the mitochondrial membrane potential. Thus, recovery of the mitochondrial membrane potential is an important reason explaining NO's protection against oxidative stress-induced osteoblast apoptosis.

Cytochrome *c* is an apoptotic protein.[34] The release of cytochrome *c* from mitochondria to the cytoplasm can trigger apoptosis in osteoblasts.[35] Oxidative stress increased the cellular levels of cytochrome *c* protein. Such an increase put osteoblasts at a high risk of cell apoptosis. Cytochrome *c* can be released due to depolarization of the mitochondrial membrane potential induced by oxidative stress. After pretreatment with NO, levels of cytochrome *c* in the osteoblasts were significantly lower. Thus, NO pretreatment suppressed the oxidative stress-caused cytochrome *c* release, and decreased the percentage of osteoblasts undergoing apoptosis.

The release of cytochrome *c* increases the binding frequency of this apoptotic protein to procaspase protease family members, leading to the sequential decomposition of this enzyme into activated subunits.[36] In parallel with the release of cytochrome *c*, oxidative stress increased the activity of caspase-3. Activation of caspase-3 can cause the digestion of key cellular proteins and induce DNA fragmentation and cell apoptosis.[36,37] Thus, oxidative stress induces osteoblast apoptosis via a cytochrome *c*-mediated activation of caspase-3 activity and DNA fragmentation. Pretreatment with NO significantly decreases the oxidative stress-involved augmentation of caspase-3 activity and DNA fragmentation. Therefore, NO pretreatment decreases oxidative stress-induced caspase-3 activation and DNA fragmentation.

In summary, SNP at a low concentration of 0.3 mM was decomposed into low levels of NO without influencing cell viability. However, SNP at 2 mM caused oxidative stress to osteoblasts and induced cellular insults. Pretreatment of osteoblasts with NO for 24 h significantly decreased the oxidative stress-induced cell injuries via an antiapoptotic mechanism. NO pretreatment suppressed oxidative stress-caused increases in Bax protein synthesis and cytochrome *c* release. After pretreatment with NO, the oxidative stress-induced reduction in the mitochondrial membrane potential decreased. Sequentially, caspase-3 activity and DNA fragmentation induced by oxidative stress were also suppressed following pretreatment with NO. Therefore, this study has shown that NO pretreatment can protect osteoblasts from oxidative stress-induced apoptotic insults. The protective mechanism may involve a mitochondria-dependent pathway.

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