Pretreatment with Low Nitric Oxide Protects Osteoblasts from High Nitric Oxide-Induced Apoptotic Insults through Regulation of c-Jun N-Terminal Kinase/c-Jun-Mediated *Bcl-2* Gene Expression and Protein Translocation

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ABSTRACT: Nitric oxide (NO) can regulate osteoblast activity. In this study, we evaluated the effects of pretreatment with a low concentration of NO on osteoblast injuries induced by a high level of NO and its possible molecular mechanisms. Exposure of osteoblasts to 0.3 mM sodium nitroprusside (SNP), an NO donor, slightly increased cellular NO levels without affecting cell viability. SNP at 2 mM greatly increased the levels of cellular NO and reactive oxygen species, and induced osteoblast death. Thus, osteoblasts were treated with 0.3 and 2 mM SNP as the sources of low and high NO, respectively. Exposure of osteoblasts to high NO decreased alkaline phosphatase (ALP) activity and cell viability, and induced cell apoptosis. With low-NO pretreatment, the high NO-induced cell insults were significantly ameliorated. When the culture medium was totally replaced after pretreatment with low NO, the protective effects obviously decreased. Administration of high NO significantly decreased c-Jun N-terminal kinase (JNK) phosphorylation and nuclear c-Jun levels. Meanwhile, pretreatment with low NO significantly alleviated the high NO-induced reduction in activation of JNK and c-Jun. Sequentially, high NO inhibited Bcl-2 mRNA and protein synthesis. After pretreatment with low NO, the high NO-induced inhibition of the production of Bcl-2 mRNA and protein significantly decreased. Imaging analysis from confocal microscopy further revealed that high NO decreased translocation of the Bcl-2 protein from the cytoplasm to mitochondria. However, pretreatment with low NO significantly ameliorated the high NO-induced suppression of Bcl-2′s translocation. Exposure of human osteoblasts to high NO significantly decreased ALP activity and cell viability, and induced cell apoptosis. Pretreatment with low NO significantly lowered the high NO-induced alterations in ALP activity, cell viability, and cell apoptosis. This study shows that pretreatment with low NO can protect osteoblasts from high NO-induced cell insults via JNK/c-Jun-mediated regulation of *Bcl-2* gene expression and protein translocation. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:625–635, 2007

Keywords: nitric oxide; osteoblasts; protection; JNK/c-Jun activation; Bcl-2

INTRODUCTION

Nitric oxide (NO) is a pleiotropic signaling molecule with crucial roles in regulating osteoblast functions.[1] Constitutive NO contributes to the maintenance of osteoblast activity.[2–4] Meanwhile, during inflammation, NO can be overproduced, which harms osteoblasts.[5,6] Our previous studies showed that overproduction of NO from exogenous or endogenous sources induces osteoblast apoptosis via a mitochondrion-dependent mechanism.[5–7] In inflammation-induced osteoporosis, elevated levels of NO were shown to induce osteoblast apoptosis, and to decrease bone mineral density.[8] Endogenous NO has been shown to play an important role in regulating osteoblast activities.[9,10] Therefore, NO has biphasic effects on osteoblast functions. Constitutive NO can regulate bone function through modulating the proliferation and differentiation of osteoblasts. However, higher amounts of NO can lead to cell insults and bone defects.

NO has been implicated as having protective roles in oxidative stress-induced cell damage.[1,2] In oxidative stress-induced neuronal cell death,

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NO can upregulate the antioxidant thioredoxin system to prevent neurons from dying.[11] NO exerts an antiapoptotic role in hepatectomized mice.[12] In ischemia and reperfusion injury, NO preconditioning was shown to produce protective effects on cardiomyocytes, hepatocytes, and endothelial cells.[13] NO produced by endothelial NO synthase can mediate estrogen protection against ovariectomy-induced bone loss in rats.[14] During inflammation, cellular oxidative stress is enhanced and places osteoblasts at a high risk of cell injury.[15] Our previous studies showed that high levels of NO cause oxidative stress to osteoblasts, leading to cell dysfunction or even death.[5,16] The oxidative stress-induced osteoblast insults can be ameliorated following pretreatment with low levels of NO.[17] However, the signal transducing-mechanisms of low NO's protection against high NO-induced osteoblast damage are still not fully known.

Bcl-2 and Bax are two apoptosis-related proteins. Activation of Bax is essential for triggering cytochrome c release and cell apoptosis.[18] Bcl-2 can prevent Bax-induced mitochondrial apoptosis by blocking the release of cytochrome c.[19] A previous study showed that Bcl-2-mediated antiapoptosis is associated with c-Jun N-terminal kinase (JNK) activation.[20] Phosphorylation of Bcl-2 activated by mitogen-activated protein kinases has been verified to stimulate translocation of this antiapoptotic protein from the cytoplasm to mitochondria.[11] In addition, overexpression of Bcl-2 has been shown to stabilize mitochondria and inhibit cell death.[21] c-Jun, a transcription factor activated by JNK, contributes to regulation of *Bcl-2* gene expression.[22] In this study, we evaluated the effects of pretreatment with low NO on high NO-induced osteoblast insults using neonatal rat calvarial osteoblasts as the experimental model and investigated its possible signal-transducing mechanisms.

MATERIALS AND METHODS

Preparation of Rat Osteoblasts and Drug Treatment

Rat osteoblasts were prepared from 3-day-old Wistar rat calvaria according to a previously described method.[23] The procedures were pre-approved by the Institutional Animal Care and Use Committee of Taipei Medical University. A human osteosarcoma cell line, MG63, was purchased from American Type Culture Collection (Rockville, MD). Osteoblasts were seeded in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% 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.

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 studies showed that exposure of osteoblasts to 0.3 mM SNP significantly increased cellular NO levels but did not change cell viability. Meanwhile, 2 mM SNP caused oxidative stress and induced cell damage. Thus, SNP at 0.3 and 2 mM was used as the sources of low and high NO, respectively.

Quantification of Cellular NO and Intracellular Reactive Oxygen Species (ROS)

Levels of cellular NO were determined by the Griess reaction as described previously.[24] After drug treatment, the culture medium was collected and centrifuged. Following a reaction of the supernatant with sulfanilamide and *N*-1-napthylethylenediamine, a colorimetric azo compound was formed and quantified using a microplate photometer (Anthos Labtec Instruments, Lagerhausstrasse, Wales, Austria).

Amounts of intracellular ROS were quantified following a previously described method.[25] Briefly, 5×10^5 osteoblasts were cotreated with SNP and 2′7′-dichlorofluorescin diacetate, an ROS-sensitive dye. After drug treatment, osteoblasts were suspended in PBS buffer. Relative fluorescent intensities were quantified using a flow cytometer (Becton Dickinson, San Jose, CA).

Assays of Alkaline Phosphatase Activity (ALP) and Cell Viability

ALP activity was determined by detecting the formation of *p*-nitrophenol following a colorimetric procedure provided by the Sigma Diagnostics Alkaline, Acid and Prostatic Acid Phosphatase kit (Sigma). The ALP results were adjusted using the total protein levels.

Cell viability was determined by a trypan blue exclusion method.[26] Briefly, osteoblasts (2×10^5 cells) were cultured in 24-well tissue culture plates. After drug administration, cells were trypsinized by 0.1% trypsin-EDTA (Gibco-BRL). Following centrifugation and washing, osteoblasts were suspended in PBS and stained with an equal volume of trypan blue dye (Sigma). Fractions of dead cells with a blue signal were visualized and counted using a reverse-phase microscope (Nikon, Tokyo, Japan).

Immunodetection of JNK and Phosphorylated JNK

After drug treatment, 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]. Protein concentrations were quantified using a bicinchonic acid protein assay kit (Pierce, Rockford, IL). The proteins (100 μg per well) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. After blocking, phosphorylated JNK was immunodetected using a rabbit polyclonal antibody with a synthetic phospho-peptide

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corresponding to residues Thr183/Tyr185 of human JNK (Cell Signaling, Danvers, MA). JNK was detected using a mouse monoclonal antibody against human JNK (Cell Signaling) as the internal standard. These protein bands were quantified using a digital imaging system (UVtec, Cambridge, UK).

Preparation of Nuclear Protein and Immunoblot Analyses

After drug treatment, nuclear extracts of osteoblasts were prepared. Protein concentrations were quantified by a BCA protein assay kit (Pierce). Nuclear proteins (50 μg/well) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. After blocking, nuclear c-Jun was immunodetected using a rabbit polyclonal antibody against mouse c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA). Proliferating cell nuclear antigen (PCNA) was detected using a mouse monoclonal antibody against rat PCNA protein (Santa Cruz Biotechnology) as the internal standard. Intensities of the immunoreactive bands were determined using a digital imaging system (UVtec).

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Assay

Messenger RNA from control and SNP-treated osteoblasts was prepared for RT-PCR analyses of Bcl-2 and β-actin mRNA according to a previously described method.[27] Oligonucleotides for PCR analyses of Bcl-2 and β-actin were designed and synthesized by Clontech Laboratories (Palo Alto, CA). The oligonucleotide sequences of upstream and downstream primers for these mRNA analyses were, respectively, 5′-CAAGAATGCAAAGCACATCC-3′ and 5′-ATCCCAGCCTCCGTTATCC-3′ for Bcl-2 and 5′-TATGGAGAAGATTTGGCACC-3′ and 5′-CCACCAATCCACACAGAGTA-3′ for β-actin.[28] The PCR products were loaded and separated in 1.8% agarose gels containing 0.1 μg/ml ethidium bromide.

Confocal Microscopic Analysis of Translocation of the Bcl-2 Protein

The Bcl-2 protein in osteoblasts was recognized by a specific antibody, and visualized using confocal microscopy. Briefly, after drug treatment, osteoblasts were fixed with a fixing reagent (acetone:methanol = 1:1) at −20°C for 10 min. Following rehydration, cells were incubated with 0.2% Triton X-100 at room temperature for 15 min. Immunodetection of Bcl-2 protein in osteoblasts was carried out by reacting it with a mouse monoclonal antibody against human Bcl-2 (Santa Cruz Biotechnology) at 4°C overnight. After washing, osteoblasts were sequentially reacted with the second antibodies and biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 h. After washing, the third antibody with Cy3-conjugated streptavidin (Jackson ImmunoResearch) was added to cells and reacted at room temperature for 30 min. Mitochondria of osteoblasts were stained with 3,3′-dihexyloxacarbocyanine ($DiOC_6$) (Molecular Probes) at 37°C for 30 min. A confocal microscope (Olympus, Tokyo, Japan) was utilized for sample observation. Images were acquired using FLUOVIEW software (Olympus).

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 <0.05. Statistical analysis between drug-treated groups was carried out using two-way ANOVA.

RESULTS

Pretreatment of osteoblasts with 0.3 mM SNP for 24 h enhanced cellular NO and ROS levels by 30% and 57%, respectively (Fig. 1). However, exposure to 2 mM SNP for 1, 3, 6, 12, and 24 h caused significant increases in cellular NO amounts by 2.4-, 3.8-, 5.2-, 6.6-, and 8.2-fold, and in intracellular ROS levels by 2.5-, 4.3-, 7.5-, 8-, and 10.5-fold. When osteoblasts were pretreated with 0.3 mM SNP and then exposed to 2 mM SNP, cellular NO and ROS levels were synergistically augmented (Fig. 1). Pretreatment with 0.3 mM SNP did not affect cell viability (Fig. 2). Meanwhile, SNP at 2 mM caused a significant amount of cells to die. Thus, osteoblasts were exposed to 0.3 and 2 mM SNP as the sources of low and high NO, respectively.

ALP activity, cell viability, and apoptotic cells were further assayed to determine the protective effects of pretreatment with low NO on high NO-induced osteoblast insults (Fig. 2). After pretreatment with low NO for 24 h, the culture medium was not removed, and the high NO-induced reagent was directly added to treat osteoblasts for another 24 h (Fig. 2A, C, E). Under these treatment conditions, high NO decreased ALP activity by 78% and cell viability by 94%, and induced cell apoptosis by 85%. Pretreatment with low NO caused no discernible cell damage. However, after pretreatment with low NO, the high NO-induced alternations in ALP activity, cell viability, and cell apoptosis were significantly decreased by 39%, 47%, and 61%, respectively. In comparison, after pretreatment with low NO for 24 h, the culture medium was totally removed, and new medium containing the high NO-induced reagent was added to the osteoblasts for another 24 h (Fig. 2B, D, F). Under such treatment conditions, high NO decreased ALP activity by 81% and cell viability by 95%, and

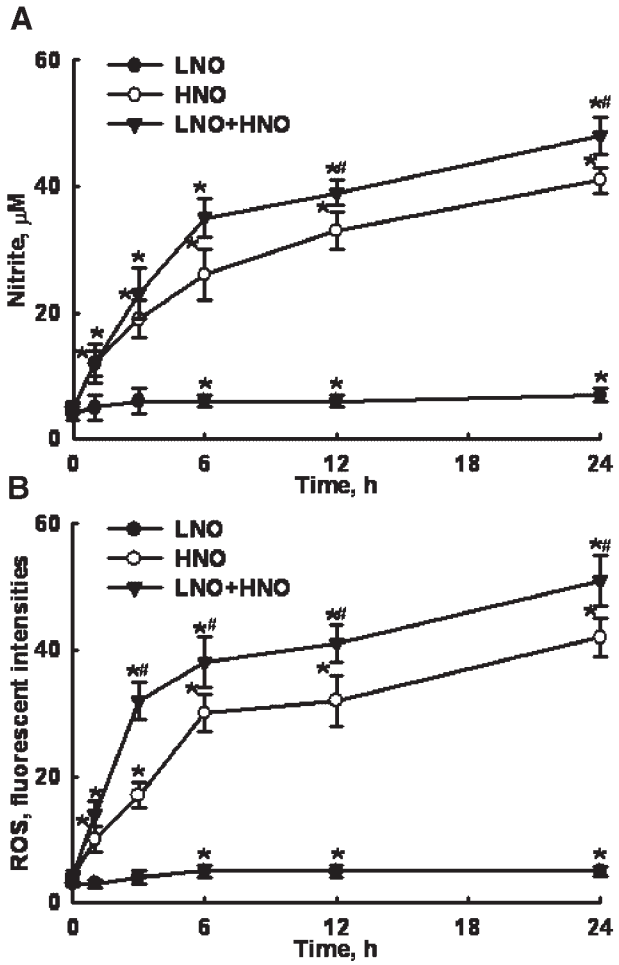

Figure 1. Effects of sodium nitroprusside (SNP) on cellular nitric oxide (NO) and intracellular reactive oxygen species (ROS) levels. Osteoblasts prepared from neonatal rat calvaria were exposed to 0.3 and 2 mM SNP as the sources of low (LNO) and high NO (HNO). Osteoblasts were treated with LNO, HNO, and a combination of LNO pretreatment and then HNO exposure for 1, 3, 6, 12, and 24 h. Cellular NO levels were determined using the Griess reaction (A). Amounts of intracellular ROS were quantified using flow cytometry (B). Each value represents the mean ± SEM, $n = 4$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and HNO-treated groups, respectively.

induced cell apoptosis by 91%. Pretreatment with low NO did not lead to osteoblast dysfunction. Compared to Figure 2A, C, and E, pretreatment with low NO significantly ameliorated high NO-induced changes in ALP activity, cell viability, and cell apoptosis by only 18%, 6%, and 20%, respectively.

To evaluate the molecular mechanism of NO's protection against osteoblast insults, the cellular levels of JNK and its phosphorylated form were immunodetected (Fig. 3A, B). Subjecting osteoblasts to high NO decreased the levels of phosphorylated JNK in osteoblasts (Fig. 3A, top panel, lane 2). Pretreatment with low NO did not change cellular JNK activation (lane 3). After pretreatment with low NO, the high NO-induced reduction in cellular JNK phosphorylation obviously decreased (Fig. 3A, lane 4). The amounts of JNK were immunodetected as the internal standard (Fig. 3A, bottom panel). These immunoreactive protein bands were quantified and analyzed (Fig. 3B). Administration of high NO to osteoblasts significantly decreased the levels of phosphorylated JNK by 67%. Pretreatment with low NO caused a complete recovery in high NO-induced decreases in phosphorylated JNK levels.

Levels of nuclear c-Jun were detected to determine the signal-transducing mechanism of NO's protection of osteoblasts (Fig. 3C, D). Exposure to high NO reduced the nuclear levels of c-Jun (Fig. 3C, top panel, lane 2). Pretreatment with low NO did not change the amount of nuclear c-Jun (Fig. 3C, lane 3). After pretreatment with low NO, the high NO-induced reduction in nuclear c-Jun production completely recovered (Fig. 3C, lane 4). Nuclear PCNA proteins were immunodetected as the internal standard (Fig. 3C, bottom panel). These protein bands were quantified and analyzed (Fig. 3D). Exposure to high NO significantly decreased the levels of nuclear c-Jun by 47%. However, pretreatment with low NO significantly lowered the large NO-induced decreases in nuclear c-Jun levels by 100% (Fig. 3D).

To further verify the sequential mechanism of low NO's protection of osteoblasts, *Bcl-2* gene expression was determined (Fig. 4). The administration of high NO inhibited Bcl-2 mRNA synthesis in osteoblasts (Fig. 4A, top panel, lane 2). Pretreatment with low NO did not influence cellular Bcl-2 mRNA levels (Fig. 4A, lane 3). After pretreatment with low NO, the high NO-induced inhibition of Bcl-2 mRNA production was significantly ameliorated (Fig. 4A, lane 4). Amounts of β-actin mRNA in osteoblasts were quantified as the internal standard (Fig. 4A, bottom panel). These DNA bands were quantified and analyzed (Fig. 4B). High NO inhibited Bcl-2 mRNA synthesis in osteoblasts by 56%. Pretreatment with low NO allowed significant recovery of the high NO-induced suppression of Bcl-2 mRNA production. Bcl-2 and β-actin were immunodetected, and these protein bands were quantified and analyzed (Fig. 4C, D). Bcl-2 protein was detected in osteoblasts (Fig. 4C, lane 1, 4D). Exposure to high

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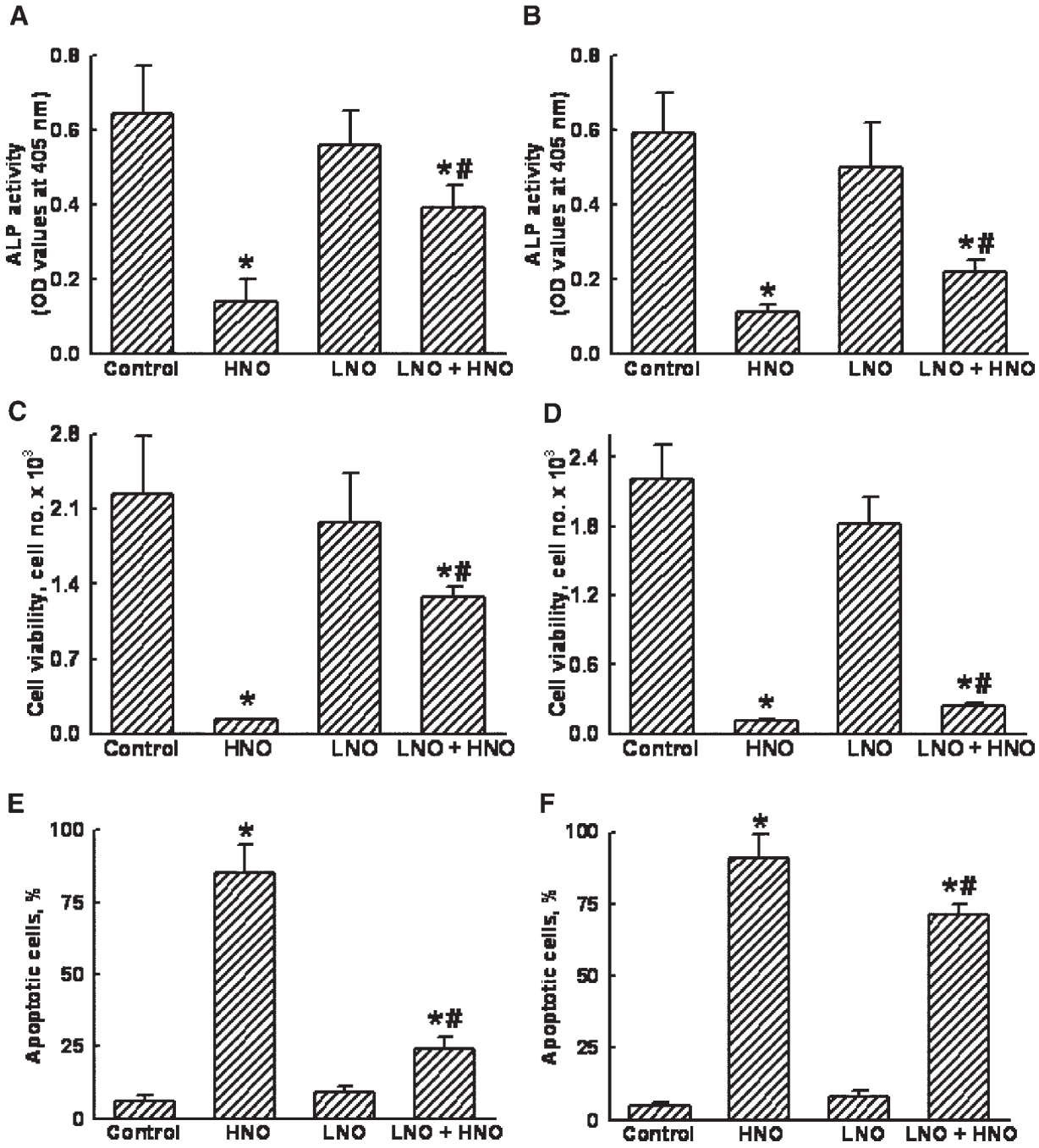

Figure 2. Protective effects of pretreatment with low nitric oxide (LNO) against high NO (HNO)-induced osteoblast insults. Osteoblasts prepared from neonatal rat calvaria were exposed to 0.3 and 2 mM sodium nitroprusside as the sources of LNO and HNO. Osteoblasts were pretreated with LNO for 24 h. After LNO pretreatment, the culture medium was retained (A, C, and E) or totally removed (B, D, and F), and osteoblasts were exposed to HNO for a further 24 h. ALP activity (A and B) and cell viability (C and D) were assayed by a colorimetric method and a trypan blue exclusion analysis, respectively. Apoptotic cells were quantified using flow cytometry (E and F). Each value represents the mean $\pm$ SEM, $n = 4$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and HNO-treated groups, respectively.

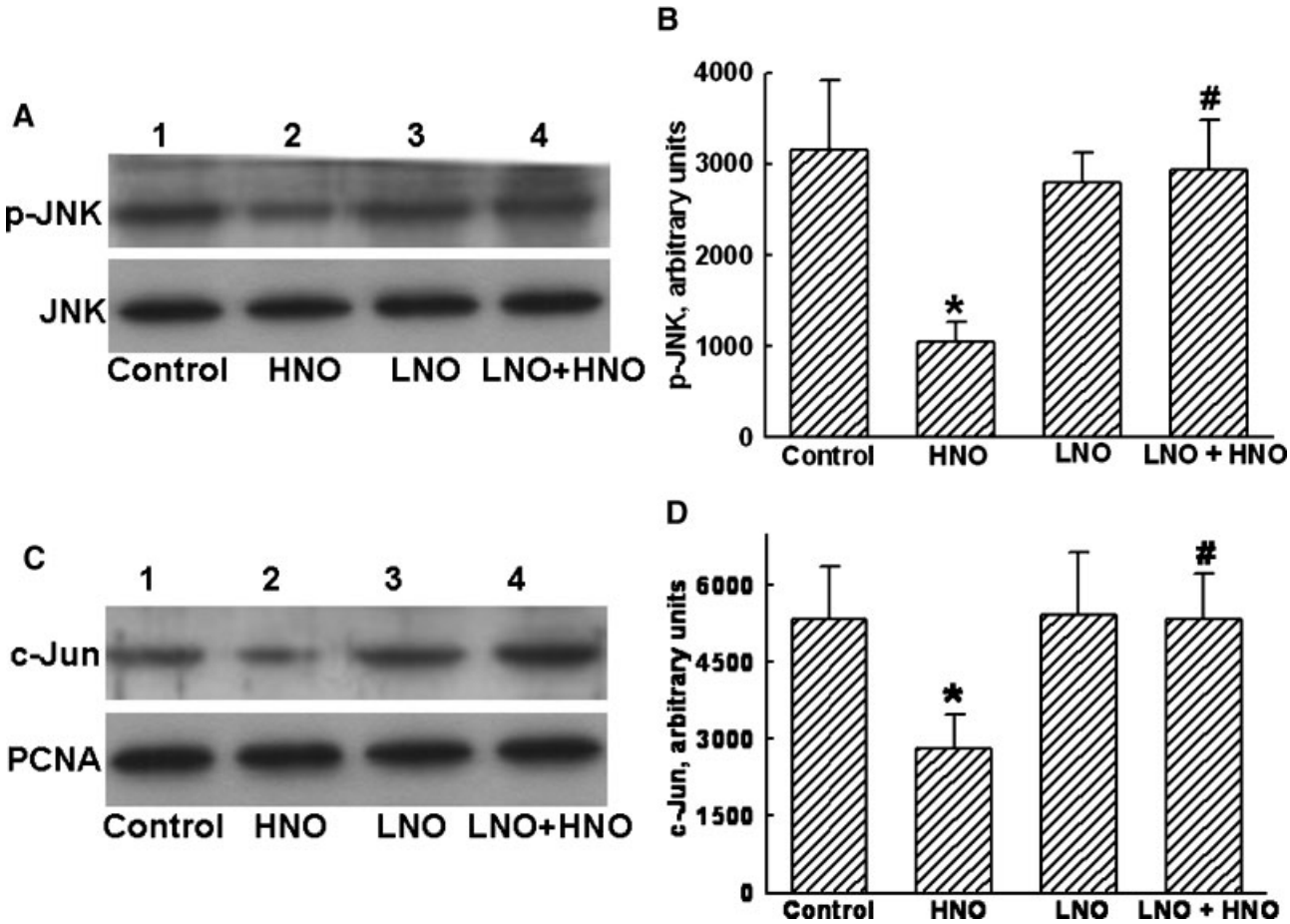

Figure 3. Effects of pretreatment with low nitric oxide (LNO) on high NO (HNO)-induced inhibition of JNK and c-Jun activation. Osteoblasts prepared from neonatal rat calvaria were exposed to 0.3 and 2 mM sodium nitroprusside as the sources of LNO and HNO. Osteoblasts were pretreated with LNO for 24 h, and then exposed to HNO for a further 1 h. Phosphorylated JNK (p-JNK) and nuclear c-Jun were immunodetected (A and C, top panels). JNK and PCNA were detected as internal controls (A and C, bottom panels). The intensities of these protein bands were quantified and analyzed (B and D). Each value represents the mean ± SEM, $n = 4$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and HNO-treated groups.

NO significantly decreased Bcl-2 synthesis by 69% (Figs. 4C, lane 2, 4D). Pretreatment with low NO did not change cellular Bcl-2 protein levels (Fig. 4C, lane 3, 4D). After pretreatment with low NO, the high NO-induced suppression of Bcl-2 production was significantly reversed (Fig. 4C, lane 4, 4D).

Imaging analysis of Bcl-2′s distribution in osteoblasts was carried out using confocal microscopy to evaluate the effects of pretreatment with low NO on the translocation of this antiapoptotic protein from the cytoplasm to mitochondria (Fig. 5). Exposure to high NO decreased the translocation of Bcl-2 protein from the cytoplasm to mitochondria. Pretreatment with low NO obviously increased Bcl-2′s translocation to mitochondria. The translocation of Bcl-2 protein decreased by high NO treatment was restored after pretreatment with low NO (Fig. 5).

The protective effects of NO on human osteoblasts were determined (Fig. 6). Exposure of human osteosarcoma MG63 cells to high NO decreased ALP activity by 71% and cell viability by 94%, and induced cell apoptosis by 69% (Fig. 6A–C). Pretreatment with low NO did not affect ALP activity, cell viability, or cell apoptosis. After pretreatment with low NO, the high NO-induced alterations in ALP activity, cell viability, and cell apoptosis were significantly decreased by 45%, 78%, and 62%, respectively (Fig. 6).

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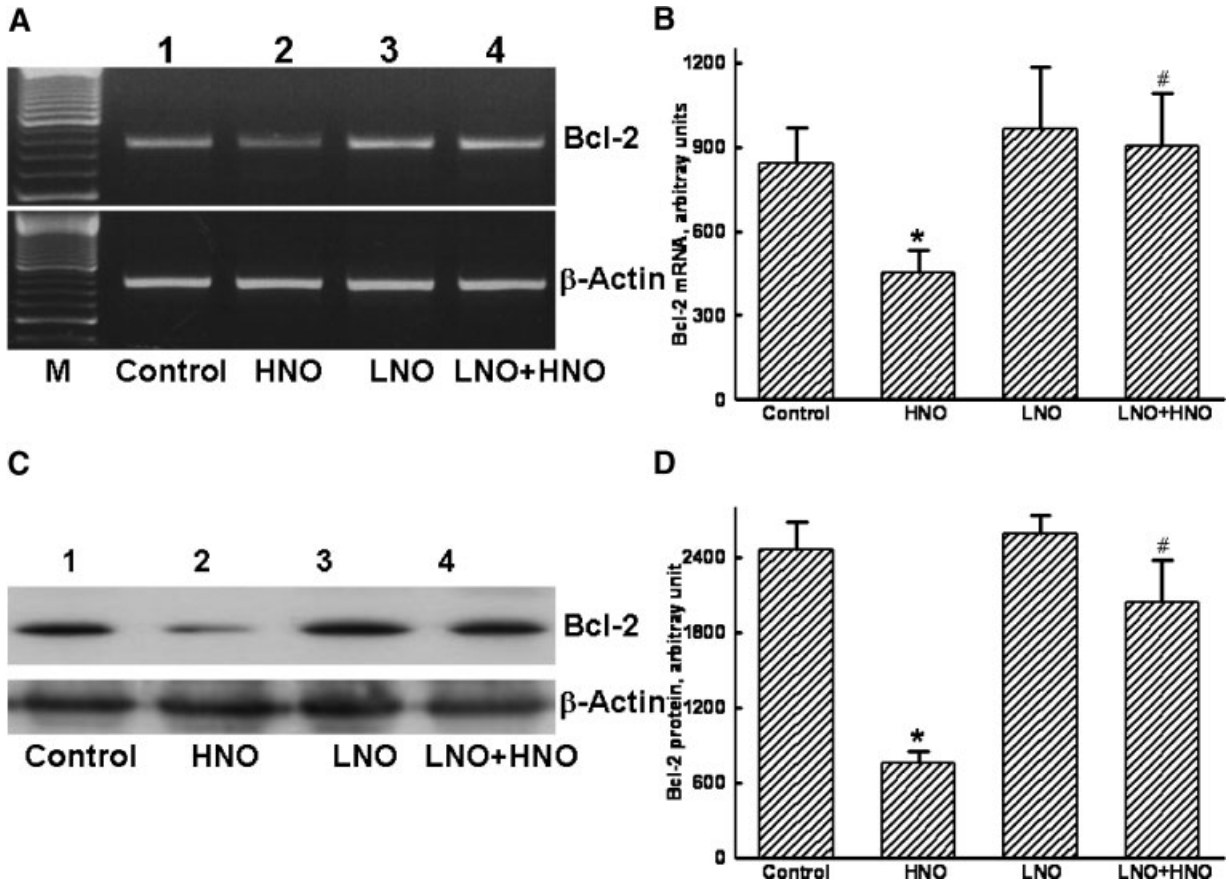

Figure 4. Effects of pretreatment with low nitric oxide (LNO) on high NO (HNO)-induced suppression of Bcl-2 mRNA and protein synthesis. Osteoblasts prepared from neonatal rat calvaria were pretreated with LNO for 24 h, and then exposed to HNO for a further 6 or 16 h. Levels of Bcl-2 mRNA and protein in osteoblasts were determined by analyses of RT-PCR and immunoblotting, respectively (A and C, top panels). Amounts of β-actin mRNA and protein were respectively detected as the internal standards (A and C, bottom panels). These DNA and protein bands were quantified and analyzed (B and D). Each value represents the mean ± SEM, $n = 4$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and HNO-treated groups.

DISCUSSION

High NO induces oxidative stress and osteoblast dysfunction. The sources of cellular oxidative stress to osteoblasts can be from NO itself, which is decomposed from SNP when exposed to light or under a biological reducing system, or from NO's derivative, peroxynitrite, an oxidative product with superoxide.[29] These oxidants enhance cellular oxidative stress in osteoblasts and decrease ALP enzyme activity. ALP is a representative enzyme in osteoblasts.[2] A decrease in ALP activity means that high NO has caused osteoblast dysfunction. In addition, our previous studies showed that overproduction of NO suppresses the mitochondrial membrane potential, complex I NADH dehydrogenase activity, and cellular ATP synthesis.[5,7] Accompanied by the reduction in ALP activity, exposure to high NO significantly decreases osteoblast viability and induces cell apoptosis. Therefore, NO released from SNP decomposition can cause oxidative stress to osteoblasts, leading to cell dysfunction and even apoptosis.

Pretreatment with low NO can protect osteoblasts from high NO-induced cellular insults. The high NO-induced cell apoptosis and reduction in ALP activity and cell viability were significantly ameliorated after pretreatment with low NO. In human osteoblasts, pretreatment with NO can also protect cells from high NO-induced insults. The present results show that pretreatment with low NO synergistically enhanced the high NO-induced increases in cellular NO and ROS levels. Thus,

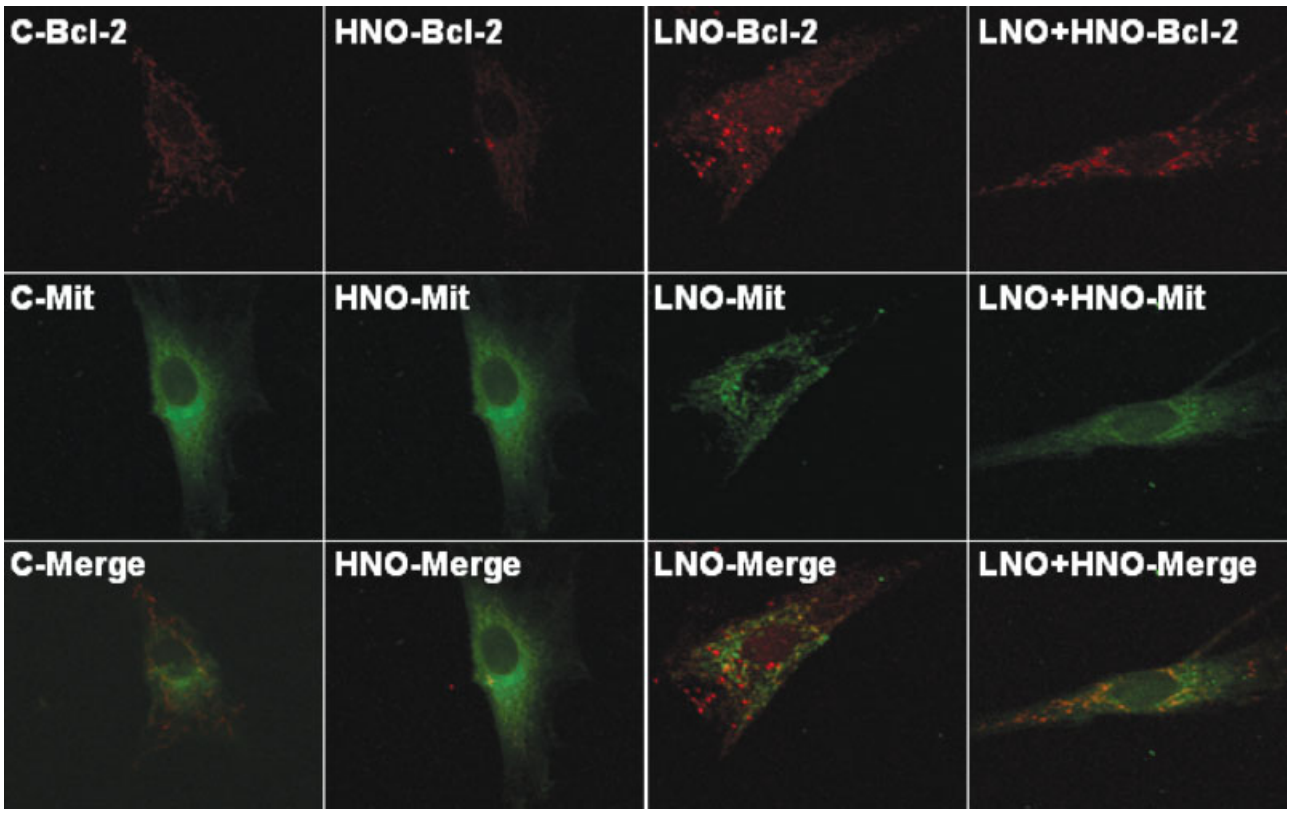

Figure 5. Effects of low nitric oxide (LNO) and high NO (HNO) on translocation of the Bcl-2 protein. Osteoblasts prepared from neonatal rat calvaria were pretreated with LNO for 24 h, and then exposed to HNO for a further 6 h. The distribution of Bcl-2 protein in osteoblasts was immunodetected. The mitochondria of osteoblasts were stained with $DiOC_6$, a positively charged dye. Fluorescent images were visualized using a confocal laser scanning microscope.

NO's protection of osteoblasts might not be due to the suppression of cellular oxidative stress. In other cell types, low NO has been reported to prevent stress-induced cell injuries.[12–14] In this study, we further showed that pretreatment with low NO did not cause osteoblast dysfunction, but lowered the high NO-induced alterations in ALP activity, cell viability, cell apoptosis, activation of JNK and c-Jun, and regulation of *Bcl-2* gene expression and protein translocation. Osteoblasts play key roles in bone remodeling.[2] During inflammation, oxidative stress can damage osteoblast functions and cause imbalances in bone remodeling, leading to bone diseases.[6,8] Therefore, NO pretreatment may be beneficial in preventing bone diseases through reducing the loss of osteoblasts.

After pretreatment with low NO, retention of the original culture medium is essential for NO's protective action against osteoblast damage. In this study, our results revealed that when the culture medium pretreated with low NO was retained and then the high NO-inducing agent was added to the osteoblasts, pretreatment with low NO significantly lowered the high NO-induced decreases in ALP activity and cell viability by 39% and 47%, respectively. In comparison, when new medium containing the high NO-causing agent was added to the osteoblasts instead of the original culture medium, pretreatment with low NO restored ALP activity and cell viability by only 18% and 6%, respectively. Thus, the culture medium pretreated with low NO is necessary to meditate NO's protection of osteoblasts. Previous studies showed that pretreatment with low NO increases cellular antioxidant proteins, including glutathione, thioredoxins, and heat shock proteins.[29,30] Therefore, one of the major explanations of the role of NO-pretreated culture medium may be that pretreatment with low NO enhances the production and release of these antioxidant proteins to the culture medium, and consequently produces protective effects on osteoblasts.

JNK activation participates in NO's protection against osteoblast insults. Exposure to high NO

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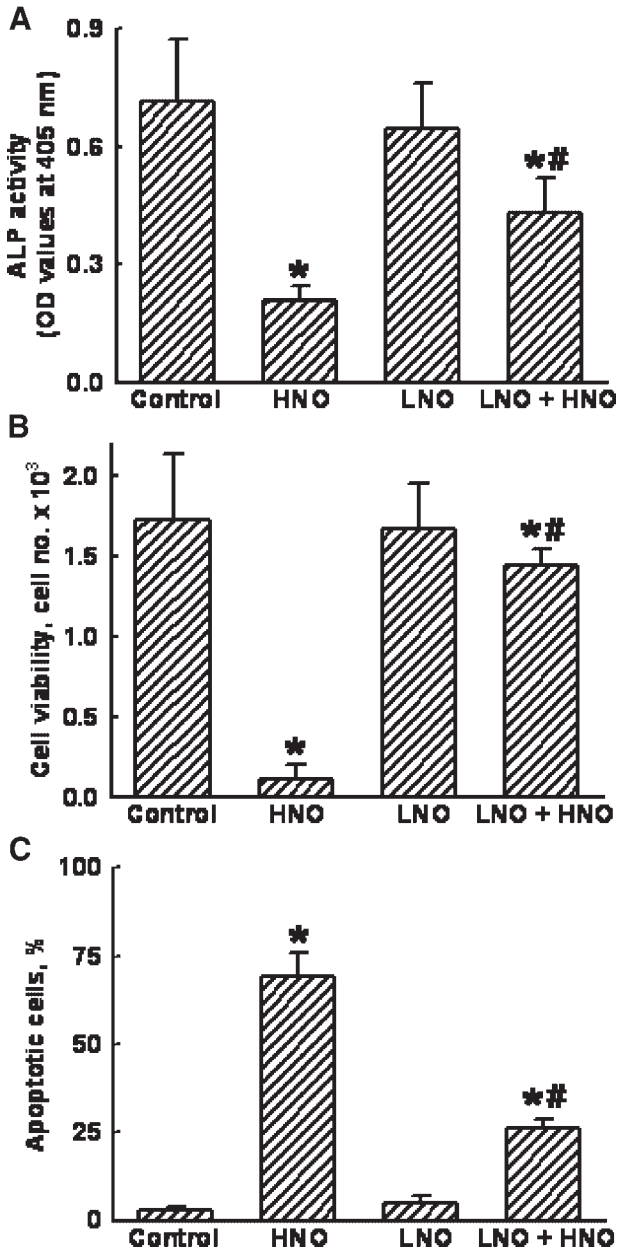

Figure 6. Protective effects of pretreatment with low nitric oxide (LNO) on high NO (HNO)-induced insults to human osteoblasts. Human osteosarcoma MG63 cells were exposed to 0.3 and 2 mM sodium nitroprusside as the sources of LNO and HNO. Human osteoblasts were pretreated with LNO for 24 h, and then exposed to HNO for a further 24 h. ALP activity was assayed using a colorimetric method (A). Cell viability was determined by the trypan blue exclusion method (B). Apoptotic cells were quantified using flow cytometry (C). Each value represents the mean ± SEM, $n = 4$. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and HNO-treated groups, respectively.

inhibits JNK activation. A previous study reported similar results of enhanced cellular ROS levels leading to perturbations in signal transduction pathways activated by protein kinases, including JNK, which consequently induce cell death.[31] Thus, high NO possibly induces osteoblast damage via inactivation of JNK. Pretreatment with NO significantly lowered the high NO-induced JNK inactivation. In parallel with the reverse effects, pretreatment with low NO significantly alleviated high NO-induced osteoblast death. There are two possible pathways involved in JNK-mediated protection. In B lymphocytes, JNK has been reported to activate the transcription factors, c-Myc and Egr-1, to promote cell survival and proliferation.[32] In addition, JNK can phosphorylate antiapoptotic proteins which stimulate their translocation from the cytoplasm to mitochondria, and prevents mitochondrial dysfunction, mitochondrial apoptotic factor release, and cell apoptosis.[33] Therefore, JNK activation may contribute to NO's protection against osteoblast damage.

c-Jun is a downstream effector mediating JNK-activated signals and NO's protection of osteoblasts. Administration of high NO in osteoblasts significantly decreased nuclear levels of phosphorylated c-Jun. c-Jun is one of the activator protein-1 members.[34] Following phosphorylation by JNK, activated c-Jun is translocated to nuclei and regulates certain gene expressions which are associated with cell proliferation and survival. A decrease in nuclear c-Jun levels represents inhibition of the expression of certain survival genes, causing cells to die.[35] This study further showed that pretreatment with low NO caused a significant recovery of the high NO-induced suppression of c-Jun activation and amelioration of osteoblast damage. In serum deprivation-induced neuron death, transforming growth factor-β released from astrocytes can phosphorylate neuronal c-Jun at Ser63 and prevent cell injuries.[36] Therefore, activation of JNK and c-Jun stimulated by pretreatment with low NO is involved in the signal-transducing events of NO's protection of osteoblasts.

Bcl-2 may be one of the crucial antiapoptotic proteins involved in NO's protection from osteoblast injuries. High NO inhibits Bcl-2 mRNA and protein production in osteoblasts. In this study, exposure to high NO significantly decreased nuclear c-Jun levels. c-Jun is a common transcription factor for regulating gene expressions.[32] JNK-mediated c-Jun activation has been shown to participate in *Bcl-2* gene regulation.[22] Thus, the high NO-involved inhibition of Bcl-2 synthesis

possible occurs at the transcriptional level. Bcl-2 is an antiapoptotic protein. When exposed to high NO, a decrease in cellular Bcl-2 levels causes osteoblast death. The high NO-induced suppression of Bcl-2 mRNA and protein synthesis was completely abolished by pretreatment with low NO. A previous study showed that overexpression of Bcl-2 inhibits cell death.[21] Therefore, pretreatment with low NO causes a complete rebound in the high NO-induced reduction in Bcl-2 mRNA production possibly via a JNK/c-Jun-mediated transcriptional mechanism. However, there are other antiapoptotic proteins, such as Bcl-X_L, which possibly contribute to NO's protection. Thus, the recovery of high NO-induced inhibition of Bcl-2 production may be a critical factor in NO's protection of osteoblasts.

An increase in Bcl-2 translocation from the cytoplasm to mitochondria in osteoblasts may be another crucial determinant of NO's protective action. Our data from the confocal microscopic analysis revealed that high NO decreased the translocation of Bcl-2 from the cytoplasm to mitochondria. Meanwhile, pretreatment with low NO significantly increased Bcl's translocation to mitochondria and lowered high NO-induced reduction in the redistribution of this antiapoptotic protein. After phosphorylated activation, Bcl-2 can be translocated to mitochondrial membranes preventing Bax-induced apoptotic events, including permeabilization of the mitochondrial membrane and release of cytochrome c.[19] Previous studies from our laboratory demonstrated that administration of SNP increases intracellular ROS levels, and subsequently enhances Bax protein synthesis in osteoblasts.[5,16] Mitogen-activated protein kinases can phosphorylate Bcl-2.[11] This study showed that pretreatment with low NO can activate JNK. Thus, pretreatment with low NO stimulates JNK-mediated translocation of Bcl-2 from the cytoplasm to mitochondria and produces protective effects against high NO-induced osteoblast insults. Meanwhile, other antiapoptotic proteins might be activated by JNK and participate in NO's protection. Therefore, JNK-mediated Bcl-2 translocation may be a crucial factor in NO's protection against osteoblast insults.

In summary, this study showed that high NO decreased ALP activity and induced osteoblast apoptosis. Pretreatment of osteoblasts with low NO for 24 h significantly reduced high NO-induced cell injuries. The culture medium of osteoblasts pretreated with low NO was found to be necessary to mediate NO's protection. After pretreatment with low NO, the high NO-induced inhibition of JNK and c-Jun activation was completely abolished. In parallel with the recovery, pretreatment with low NO significantly ameliorated high NO-induced suppression of Bcl-2 mRNA and protein synthesis. The translocation of Bcl-2 from the cytoplasm to mitochondria was simultaneously augmented after pretreatment with low NO. Pretreatment with low NO also has beneficial effects on protecting human osteoblasts from high NO-induced cellular insults. Taken together, results of this study show that pretreatment with low NO can protect osteoblasts from high NO-induced insults. The protective mechanism may be via JNK/c-Jun-mediated regulation of *Bcl-2* gene expression and protein translocation.

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