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Runx2-Mediated *bcl-2* Gene Expression Contributes to Nitric Oxide Protection Against Hydrogen Peroxide-Induced Osteoblast Apoptosis

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ABSTRACT

Nitric oxide (NO) can regulate osteoblast activities. This study was aimed to evaluate the protective effects of pretreatment with sodium nitroprusside (SNP) as a source of NO on hydrogen peroxide-induced osteoblast insults and its possible mechanisms. Exposure of human osteosarcoma MG63 cells to hydrogen peroxide significantly increased cellular oxidative stress, but decreased ALP activity and cell viability, inducing cell apoptosis. Pretreatment with 0.3 mM SNP significantly lowered hydrogen peroxide-induced cell insults. Treatment of human MG63 cells with hydrogen peroxide inhibited Bcl-2 mRNA and protein production, but pretreatment with 0.3 mM SNP significantly ameliorated such inhibition. Sequentially, hydrogen peroxide decreased the mitochondrial membrane potential, but increased the levels of cytochrome *c* and caspase-3 activity. Pretreatment with 0.3 mM SNP significantly lowered such alterations. Exposure to hydrogen peroxide decreased Runx2 mRNA and protein syntheses. However, pretreatment with 0.3 mM SNP significantly lowered the suppressive effects. Runx2 knockdown using RNA interference inhibited Bcl-2 mRNA production in human MG63 cells. Protection of pretreatment with 0.3 mM SNP against hydrogen peroxide-induced alterations in ALP activity, caspase-3 activity, apoptotic cells, and cell viability were also alleviated after administration of Runx2 small interference RNA. Thus, this study shows that pretreatment with 0.3 mM SNP can protect human MG63 cells from hydrogen peroxide-induced apoptotic insults possibly via Runx2-involved regulation of *bcl-2* gene expression. J. Cell. Biochem. 108: 1084–1093, 2009.

KEY WORDS: OSTEOBLASTS; NITRIC OXIDE; HYDROGEN PEROXIDE; Runx2; Bcl-2; APOPTOSIS

Nitric oxide (NO) contributes to the regulation of tissue/cell activities, including vasodilatation, neurotransmission, immunoresponses, and tumorigenesis [Moncada et al., 1991; Yerushalmi et al., 2005; Chen et al., 2005a]. In bone-derived cells, both constitutive and inducible NO synthases have been detected [Evans and Ralston, 1996]. NO has been implicated as being involved in physiological and pathophysiological regulation of bone tissues [Ralston, 1997; Tai et al., 2007]. For example, NO can decrease bone mineral density in inflammation-induced osteoporosis [Armour et al., 1999]. However, in cyclosporine A-treated rats, administration of L-arginine increases NO levels and prevents bone loss and bone collagen breakdown [Fiore et al., 2000]. Thus, NO has biphasic effects on bone activities. High levels of NO have cytotoxic effects which can induce bone disease [Ralston, 1997; Chen et al., 2005b]. Meanwhile, NO at low concentrations can promote bone metabolism.

Osteoblasts play critical roles in mediating bone formation and remodeling [Takeda and Karsenty, 2001; Aguila and Rowe, 2005]. A variety of systemic and local factors participate in regulation of osteoblast activities [Takeda and Karsenty, 2001; Vanderschueren et al., 2004; Chang et al., 2006]. NO and hydrogen peroxide are two typical reactive oxygen species, which can be augmented during

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bone inflammation [Collin-Osdoby et al., 1995; Symons, 1996; Tai et al., 2007]. Our previous studies showed that NO from exogenous decomposition of sodium nitroprusside (SNP) or endogenous responses to stimulation of interferon-γ and lipopolysaccharide induces osteoblast death via an apoptotic mechanism [Chen et al., 2002, 2005b]. Mitochondria have been shown to play an important role in NO-induced osteoblast apoptosis [Ho et al., 2005; Chen et al., 2005b; Chang et al., 2006]. In addition to its deadly effects, previous studies revealed that low levels of NO can protect a variety of cells from stress-induced apoptotic insults [Ha et al., 2003; Das et al., 2005; Figueroa et al., 2005]. In ischemia and reperfusion injury, NO preconditioning was shown to produce protective effects on cardiomyocytes, hepatocytes, and endothelial cells [Cottart et al., 1999]. Thus, NO preconditioning may be used for treatment of bone diseases, for example, bone fracture or chronic bone inflammation. However, the roles of NO pretreatment on osteoblast protection and its possible molecular mechanisms are still little known.

Bcl-2 and Bax are two apoptosis-related proteins. Activation of Bax is essential for triggering the disruption of the mitochondrial membrane potential, cytochrome *c* release, caspase activation, and cell apoptosis [Wei et al., 2001; Chen et al., 2002; Chang et al., 2006]. Bcl-2 can prevent Bax-induced mitochondrial apoptosis by blocking the release of cytochrome *c* [Luo et al., 1998; Chen et al., 2007]. A previous study showed that Bcl-2-mediated antiapoptosis is associated with c-Jun N-terminal kinase (JNK) activation [Brichese et al., 2004; Chen et al., 2005b]. 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 [Torcia et al., 2001]. Overexpression of Bcl-2 has been shown to stabilize mitochondria and inhibit cell death [Gonzalez-Polo et al., 2005]. Runx2, a transcription factor, has been reported in regulation of osteoblast growth and differentiation [Hawse et al., 2008]. In addition, Runx2 can regulate apoptosis via modulation of the Bcl-2 family protein expression in human osteoblasts [Eliseev et al., 2008]. Our previous study has shown that pretreatment with low concentrations of NO can protect rat osteoblasts from oxidative stress-induced apoptotic insults [Chang et al., 2006; Tai et al., 2007]. However, the roles of Runx2 in the regulation of oxidative stress-induced damage of human osteoblasts are still unknown. Thus, this study was designed to evaluate the effects of pretreatment with NO on hydrogen peroxide-induced insults to human osteoblasts and its possible molecular mechanisms, especially in Runx2-involved *bcl-2* gene regulation, using human osteosarcoma MG63 cells as the experimental model.

MATERIALS AND METHODS

CELL CULTURE AND DRUG TREATMENT

Human osteosarcoma MG63 cells, purchased from American Type Culture Collection (Rockville, MD), were cultured in DMEM medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf 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. Human MG63 cells were grown to confluence prior to drug treatment. SNP purchased from Sigma Corporation (St. Louis, MO) was freshly dissolved in a 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. Cellular NO levels were determined according to the Griess reaction method described previously [Wu et al., 2007]. Our preliminary study revealed that administration of 0.3 mM SNP in human MG63 cells significantly increased cellular NO levels without affecting cell viability (data not shown). Thus, pretreatment with 0.3 mM SNP were used as the sources of NO in this study.

DETERMINATION OF CELLULAR OXIDATIVE STRESS

Levels of intracellular reactive oxygen species were quantified to evaluate cellular oxidative stress to human MG63 cells after exposure to hydrogen peroxide according to a previously described method [Chang et al., 2005]. Briefly, human MG63 cells (5×10^5 cells per well) were cultured in 12-well tissue culture plates overnight, and then cotreated with drugs and 2′,7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR), a reactive oxygen species-sensitive dye. After drug treatment, human MG63 cells were harvested and suspended in 1× PBS buffer. Relative fluorescent intensities in human MG63 cells were quantified using a flow cytometer (Becton Dickinson, San Jose, CA).

ASSAY OF ALKALINE PHOSPHATASE ACTIVITY

After drug treatment, alkaline phosphatase (ALP) activity in human MG63 cells was determined by detecting the formation of *p*-nitrophenol, a product of *p*-nitrophenyl phosphate catalyzed by ALP, according to a previously described colorimetric procedure provided by a Sigma Diagnostics Alkaline, Acid, and Prostatic Acid Phosphotase kit (Sigma) [Chen et al., 2002].

ASSAY OF CELL VIABILITY

Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [Wu et al., 2008]. Briefly, human MG63 cells (1×10^4 cells per well) were seeded in 96-well tissue culture plates overnight. After drug treatment, human MG63 cells were cultured with new medium containing 0.5 mg/ml MTT for a further 3 h. The blue formazan products in human MG63 cells were dissolved in dimethyl sulfoxide and spectrophotometrically measured at a wavelength of 550 nm.

ANALYSIS OF APOPTOTIC CELLS

Apoptotic cells were determined using propidium iodide to detect DNA fragments in nuclei according to a previously described method [Cherng et al., 2008]. After drug treatment, the medium containing the floating cells was collected, and the attached cells were washed and trypsinized. The floating and trypsinized osteoblasts were collected in the same centrifuge tubes. After centrifugation and washing, the cell pellets were fixed in cold 80% ethanol. The fixed cells were stained with propidium iodide. Apoptotic cells were quantified by detecting the proportion of human MG63 cells arrested at subG1 phase using flow cytometry (Becton Dickinson).

ANALYSES OF REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Messenger RNA from control and drug-treated human MG63 cells was prepared for RT-PCR analyses of Bcl-2, Runx2, and β-actin mRNA. Oligonucleotides for PCR analyses of rat Bcl-2, Runx2, and β-actin were designed and synthesized by Clontech Laboratories (Palo Alto, CA). The oligonucleotide sequences of the respective upstream and downstream primers for these mRNA analyses were 5′-CAAGAATGCAAAGCACATCC-3′ and 5′-ATCCCAGCCTCCGTTATCC-3′ for Bcl-2 [Yamanaka et al., 2002], 5′-GACAGAAGCTTGATGACTCTAAACC-3′ and 5′-CTGTAATCTGACTCTGTCCTTGTG-3′ for Runx2 [Ontiveros et al., 2004], and 5′-TATGGAGAAGATTTGGCACC-3′ and 5′-TATGGAGAAGATTTGGCACC-3′ for β-actin [Chang et al., 2009]. The PCR reaction was carried out using 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min. After the PCR assay, the products were loaded and separated in a 1.8% agarose gel containing 0.1 μg/ml ethidium bromide. The intensities of the DNA bands in the agarose gel were quantified with the aid of a UVIDOCMW version 99.03 digital imaging system (UVtec Limited, Cambridge, England, UK).

IMMUNODETECTION OF Bcl-2, Runx2, CYTOCHROME *c*, AND β-ACTIN PROTEINS

Immunoblotting analyses were carried out as described previously [Lee et al., 2009]. After drug treatment, human MG63 cell were washed with a 1× PBS buffer. 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, 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, were added to the RIPA buffer. Protein concentrations were quantified by a bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL). Cytosolic proteins (100 μg/well) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to nitrocellulose membranes. These membranes were blocked with 5% non-fat milk at 37°C for 1 h. Bcl-2, cytochrome *c*, and Runx2 were immunodetected using mouse monoclonal antibodies against Bcl-2, cytochrome *c*, and Runx2, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). Cellular β-actin protein was immunodetected using a mouse monoclonal antibody against mouse β-actin (Sigma) as an internal standard. Intensities of these immunorelated protein bands were determined using a digital imaging system (UVtec Limited).

QUANTIFICATION OF THE MITOCHONDRIAL MEMBRANE POTENTIAL

The mitochondrial membrane potential was determined following the method of Chen et al. [2005a]. 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).

ASSAY OF CASPASE-3 ACTIVITY

The activity of caspase-3 was determined by a fluorogenic substrate assay as described previously [Chang et al., 2006]. 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.

Runx-2 KNOCKDOWN

Translation of Runx2 mRNA in human MG63 cells was knocked-down using an RNA interference (RNAi) method following a small interfering (si)RNA transfection protocol provided by Santa Cruz Biotechnology as described previously [Wu et al., 2008]. Runx2 siRNA was purchased from Santa Cruz Biotechnology, and is a pool of three target-specific 20–25-nt siRNAs designed to knock-down Runx2's expression. Briefly, after culturing human MG63 cells in antibiotic-free DMEM medium at 37°C in a humidified atmosphere of 5% CO_2 for 24 h, the siRNA duplex solution, which was diluted in the siRNA transfection medium (Santa Cruz Biotechnology), was added to the cells. After transfecting for 24 h, the medium was replaced with normal DMEM medium, and human MG63 cells were treated with drugs. Control cells were applied with scrambled siRNA.

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 determine the effects of hydrogen peroxide on cellular oxidative stress and osteoblast injuries, levels of intracellular reactive oxygen species, ALP activity, cell viability, and apoptotic cells were analyzed (Fig. 1). Exposure of human MG63 cells to 200 μM hydrogen peroxide for 1, 3, 6, 12, and 24 h time-dependently increased cellular oxidative stress by 57%, and two-, three-, five-, and sixfold, respectively (Fig. 1A). In parallel with such enhancement in cellular oxidative stress, ALP activities decreased by 28%, 36%, and 55% following treatment with hydrogen peroxide for 6, 12, and 24 h, respectively (Fig. 1B). When exposed to hydrogen peroxide for 6, 12, and 24 h, the viability of human MG63 cells was reduced by 30%, 38%, and 53%, respectively (Fig. 1C). Treatment of human MG63 cells with hydrogen peroxide for 6, 12, and 24 h

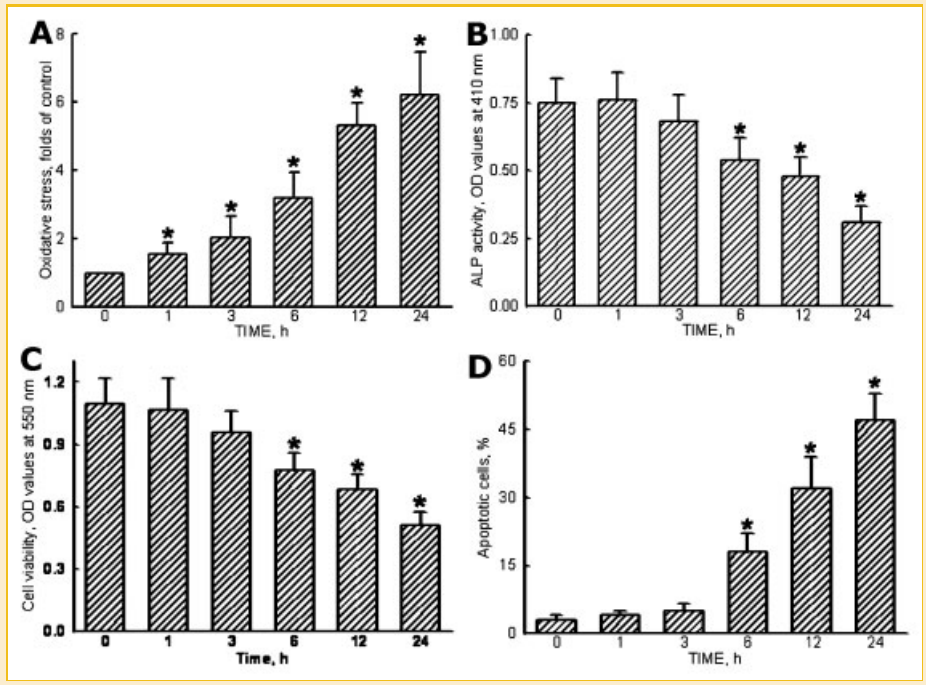

Fig. 1. Effects of hydrogen peroxide on cellular oxidative stress production and cell injuries. Human MG63 cells were treated with 200 μM hydrogen peroxide for 1, 3, 6, 12, and 24 h. Cellular oxidative stress was determined by detecting the levels of intracellular reactive oxygen species in human MG63 cells using flow cytometry (A). Alkaline phosphatase (ALP) activity (B) and cell viability (C) were analyzed according to colorimetric methods. Apoptotic cells were quantified using flow cytometry after propidium iodide staining of genomic DNA (D). Each value represents the mean ± SEM, n = 4. *Values significantly differ from the respective control, $P < 0.05$.

significantly induced 18%, 32%, and 47% of cells to undergo apoptosis (Fig. 1D).

ALP activity, cell viability, and apoptotic cells were further assayed to evaluate the effects of pretreatment with 0.3 mM SNP on hydrogen peroxide-induced osteoblast insults (Fig. 2). Exposure of human MG63 cells to hydrogen peroxide for 24 h caused a 52% decrease in ALP activity (Fig. 2A). Pretreatment with 0.3 mM SNP for 24 h did not affect ALP activity but significantly lowered the hydrogen peroxide-induced reduction in ALP activity by 57%. Hydrogen peroxide caused 50% of human MG63 cells to die (Fig. 2B). Pretreatment with 0.3 mM SNP did not cause osteoblast injury. After pretreatment with 0.3 mM SNP, the hydrogen peroxide-induced cell death was reduced by 55%. Administration of hydrogen peroxide in human MG63 cells triggered 50% of cells to undergo apoptosis (Fig. 2C). Pretreatment with 0.3 mM SNP had no affect on osteoblast apoptosis. After pretreatment with 0.3 mM SNP, the hydrogen peroxide-induced cell apoptosis was attenuated by 56%.

To validate the roles of apoptosis-related *bcl-2* gene expression in SNP's protection against hydrogen peroxide-induced osteoblast insults, analyses of RT-PCR and immunoblot were carried out on human MG63 cells (Fig. 3). In untreated human MG63 cells, Bcl-2 mRNA was detected (Fig. 3A, top panel, lane 1). The levels of Bcl-2 mRNA were inhibited following administration of hydrogen peroxide (lane 2). Pretreatment with 0.3 mM SNP did not influence Bcl-2 mRNA synthesis in human MG63 cells (lane 3). After pretreatment with NO, the hydrogen peroxide-caused inhibition of Bcl-2 mRNA dropped (lane 4). Amounts of β-actin mRNA were determined as the internal standard (Fig. 3A, bottom panel). These DNA bands were quantified and analyzed (Fig. 3B). Hydrogen peroxide inhibited Bcl-2 mRNA production by 71%. Pretreatment with 0.3 mM SNP significantly ameliorated hydrogen peroxide-induced suppression of Bcl-2 mRNA synthesis. In untreated human MG63 cells, Bcl-2 protein was immunodetected (Fig. 3C, top panel, lane 1). Exposure to hydrogen peroxide decreased cellular Bcl-2 levels (lane 2). Pretreatment with 0.3 mM SNP did not affect Bcl-2 production but significantly decreased hydrogen peroxide-caused suppression of Bcl-2 protein synthesis (lane 4). Amounts of β-actin were immunodetected as the internal standard (Fig. 3C, bottom panel). These protein bands were quantified and analyzed (Fig. 3D). Treatment of human MG63 cells with hydrogen peroxide caused a significant 68% decrease in cellular Bcl-2 levels. Pretreatment with 0.3 mM SNP significantly decreased such suppression.

The mitochondrial membrane potential, levels of cytochrome *c*, and caspase-3 activity were analyzed to determine the signal-transducing events (Fig. 4). Exposure of human MG63 cells to hydrogen peroxide caused a significant 32% decrease in the mitochondrial membrane potential (Fig. 4A). Pretreatment with 0.3 mM SNP did not affect the membrane potential of mitochondria but completely alleviated hydrogen peroxide-caused suppression. Treatment of human MG63 cells with hydrogen peroxide increased the levels of cytochrome *c* (Fig. 4B, top panel, lane 2). Pretreatment with 0.3 mM SNP lowered hydrogen peroxide-involved augmentation of cytochrome *c* (lane 4). Amounts of β-actin were immunodetected as the internal standards (Fig. 4B, bottom panel). These immunorelated protein bands were quantified and analyzed (Fig. 4C). Exposure to hydrogen peroxide significantly enhanced the

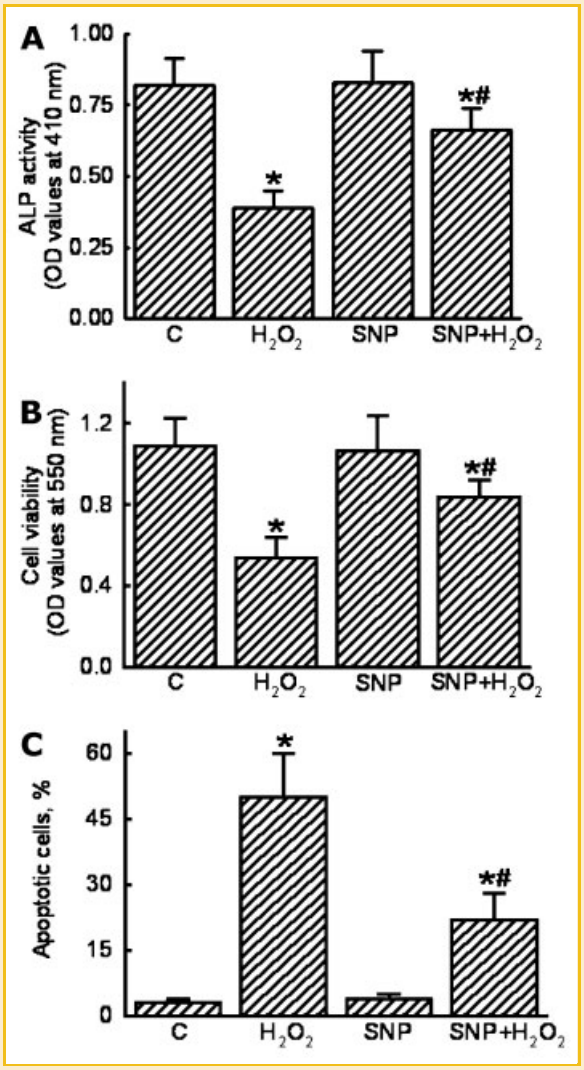

Fig. 2. Protective effects of pretreatment with 0.3 mM sodium nitroprusside (SNP) on hydrogen peroxide (H_2O_2)-induced osteoblast damage. Human MG63 cells were pretreated with 0.3 mM SNP for 24 h, and then exposed to H_2O_2 for a further 24 h. Alkaline phosphatase (ALP) activity (A) and cell viability (B) were assayed using colorimetric methods. Apoptotic cells were quantified using flow cytometry after propidium iodide staining of genomic DNA (C). Each value represents the mean ± SEM, n = 4. *Values significantly differ from the respective control, and #values have statistical signification between cells treated with H_2O_2 and with NO + H_2O_2, $P < 0.05$. C, control.

levels of cytochrome *c* by 2.1-fold. However, pretreatment with 0.3 mM SNP completely decreased such an enhancement. Activity of caspase-3 was increased by 2.2-fold following exposure to hydrogen peroxide (Fig. 4D). Pretreatment with 0.3 mM SNP did not influence caspase-3 activity, but significantly ameliorated hydrogen peroxide-caused increases in this protease activity.

RNA and protein levels of Runx2 were quantified to evaluate the roles of this transcription factor in SNP's protection (Fig. 5). In untreated human MG63 cells, Runx2 mRNA was detected (Fig. 5A, top panel, lane 1). After administration of hydrogen peroxide, Runx2 mRNA production was inhibited (lane 2). Pretreatment with 0.3 mM SNP had no effect on Runx2 mRNA synthesis (lane 3). Pretreatment with 0.3 mM SNP alleviated hydrogen peroxide-caused inhibition of Runx2 mRNA synthesis (lane 4). β-Actin mRNA in human MG63 cells was detected as the internal standard (Fig. 5A, bottom panel). These DNA bands were quantified and analyzed (Fig. 5B). Hydrogen peroxide significantly decreased Runx2 mRNA levels by 63%. Pretreatment with 0.3 mM SNP completely lowered hydrogen peroxide-induced suppression of Runx2 mRNA synthesis. Runx2 protein was immunodetected in untreated human MG63 cells (Fig. 5C, top panel, lane 1). Hydrogen peroxide decreased Runx2 protein synthesis (lane 2). Pretreatment with 0.3 mM SNP did not affect cellular Runx2 protein levels (lane 3). After pretreatment with 0.3 mM SNP, the hydrogen peroxide-induced reduction in Runx2 protein levels obviously recovered (lane 4). β-Actin protein was immunodetected as the internal control (Fig. 5C, bottom panel). These protein bands were quantified and analyzed (Fig. 5D). Hydrogen peroxide significantly decreased Runx2 protein synthesis by 64% (Fig. 5D). Pretreatment with 0.3 mM SNP significantly lowered hydrogen peroxide-induced reduction in Runx2 protein synthesis.

Translation of Runx2 mRNA was knocked-down to further evaluate the roles of this transcription factor on Bcl-2 mRNA production (Fig. 6). Application of Runx2 siRNA into human MG63 cells for 24 and 48 h markedly decreased the levels of this transcription factor (Fig. 6A, top panel). Amounts of β-actin were immunodetected as the internal controls (bottom panel). These protein bands were quantified and analyzed (Fig. 6B). Treatment of human MG63 cells with Runx2 siRNA for 24 and 48 h caused 58% and 75% decreases in the levels of this transcription factor, respectively. In parallel with this decrease, application of Runx2 siRNA inhibited Bcl-2 mRNA production (Fig. 6C, top panel, lane 1). Levels of β-actin mRNA were detected as the internal standard (bottom panel). These DNA bands were quantified and analyzed (Fig. 6D). Application of Runx2 siRNA into human MG63 cells significantly inhibited Bcl-2 mRNA synthesis by 71%.

Effects of Runx2 siRNA on protection of pretreatment with 0.3 mM SNP against hydrogen peroxide-caused alterations in ALP activity, caspase-3 activity, cell apoptosis, and cell viability were determined (Fig. 7). Treatment of human MG63 cells with Runx2 siRNA alone did not affect ALP activity, caspase-3 activity, apoptotic cells, and cell viability (Fig. 7A–D). Meanwhile, co-treatment with hydrogen peroxide and Runx2 siRNA had synergistic effects on caspase-3 activity, apoptotic cells, and cell viability (Fig. 7B–D). The protection of pretreatment with 0.3 mM SNP against hydrogen peroxide-induced insults to human MG63 cells was significantly lowered after exposure to Runx2 siRNA (Fig. 7A–D).

DISCUSSION

Hydrogen peroxide induces insults to human MG63 cells via an apoptotic mechanism. Administration of human MG63 cells with hydrogen peroxide decreased ALP activity and caused osteoblast death. ALP is one of the critical markers of osteoblast metabolism [Collin-Osdoby et al., 1995]. Thus, treatment with hydrogen peroxide can cause osteoblast dysfunction or even death. Exposure to hydrogen peroxide increased the proportion of human MG63

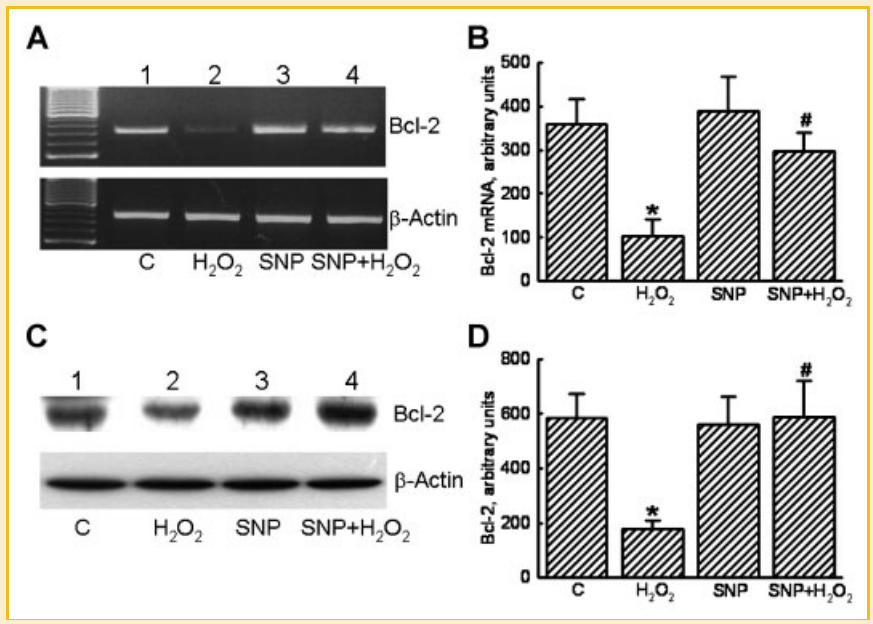

Fig. 3. Effects of pretreatment with 0.3 mM sodium nitroprusside (SNP) on hydrogen peroxide (H_2O_2)-caused inhibition of Bcl-2 mRNA and protein. Human MG63 cells were pretreated with 0.3 mM SNP for 24 h, and then exposed to H_2O_2 for a further 6 or 24 h. Levels of Bcl-2 mRNA in human MG63 cells were determined by RT-PCR analyses (A). Amounts of β-actin mRNA was quantified as the internal control. These DNA bands were quantified and analyzed (B). Levels of Bcl-2 were detected using an immunoblotting analysis (C). β-Actin was immunodetected as the internal control. These protein bands were quantified and analyzed (D). Each value represents the mean ± SEM, n = 6. *Values significantly differ from the respective control, and #values have statistical signification between cells treated with H_2O_2 and with NO + H_2O_2, $P < 0.05$. C, control.

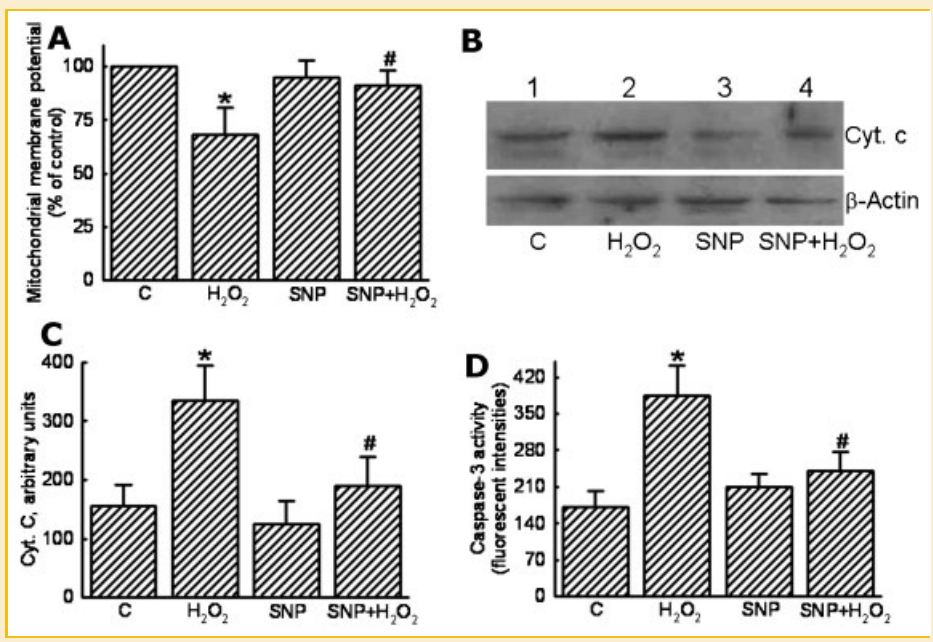

Fig. 4. Effects of pretreatment with 0.3 mM sodium nitroprusside (SNP) on hydrogen peroxide (H_2O_2)-caused alterations in the mitochondrial membrane potential, cytochrome c, and caspase-3 activity. Human MG63 cells were pretreated with 0.3 mM SNP for 24 h, and then exposed to H_2O_2. The mitochondrial membrane potential was quantified using flow cytometry (A). Levels of cytochrome c were immunodetected (B). β-Actin was determined as the internal control. These protein bands were quantified and analyzed (C). Activity of caspase-3 was analyzed using a fluorogenic substrate assay (D). Each value represents the mean ± SEM, n = 6. *Values significantly differ from the respective control, and #values have statistical signification between cells treated with H_2O_2 and with NO + H_2O_2, $P < 0.05$. C, control.

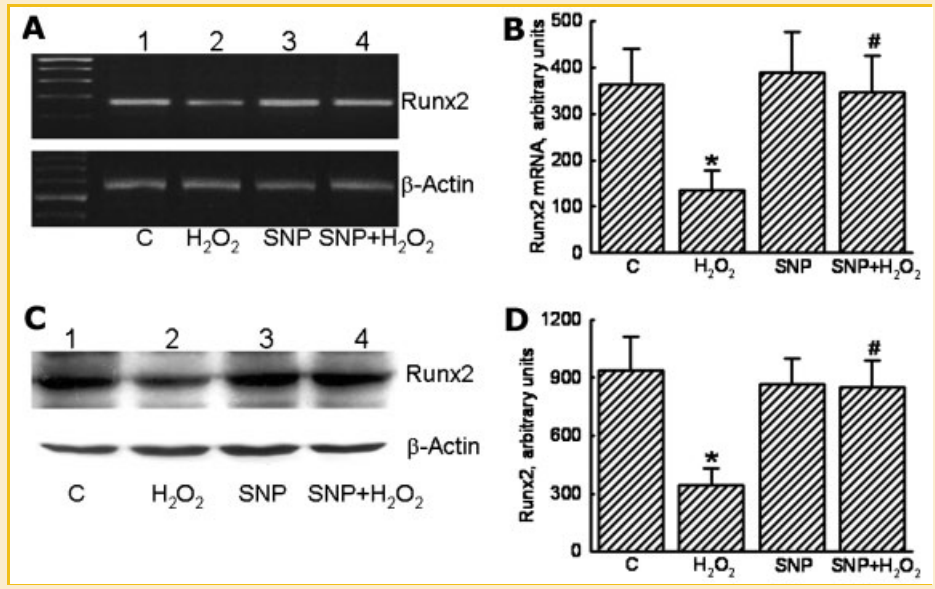

Fig. 5. Effects of pretreatment with 0.3 mM sodium nitroprusside (SNP) on hydrogen peroxide (H_2O_2)-induced suppression of Runx2 mRNA and protein production. Human MG63 cells were pretreated with 0.3 mM SNP for 24 h, and then exposed to H_2O_2 for a further 6 or 24 h. Levels of Runx2 and β-actin mRNA in human MG63 cells were determined by RT-PCR analyses (A), and these DNA bands were quantified and analyzed (B). Runx2 and β-actin proteins were immunodetected using immunoblotting analyses (C), and these protein bands were quantified and analyzed (D). Each value represents the mean ± SEM, n = 6. *Values significantly differ from the respective control, and #values have statistical signification between cells treated with H_2O_2 and with NO + H_2O_2, $P < 0.05$. C, control.

undergoing apoptosis. Our previous studies provided several lines of evidence to show that high oxidative stress can induce osteoblast shrinkage and DNA fragmentation [Liu et al., 2001; Chen et al., 2002, 2005b]. The other study executed in our lab showed that ascorbic acid could protect osteoblasts from hydrogen peroxide-induced apoptotic insults (data not shown). Therefore, the hydrogen peroxide-induced osteoblast injuries occur mainly via an apoptotic mechanism.

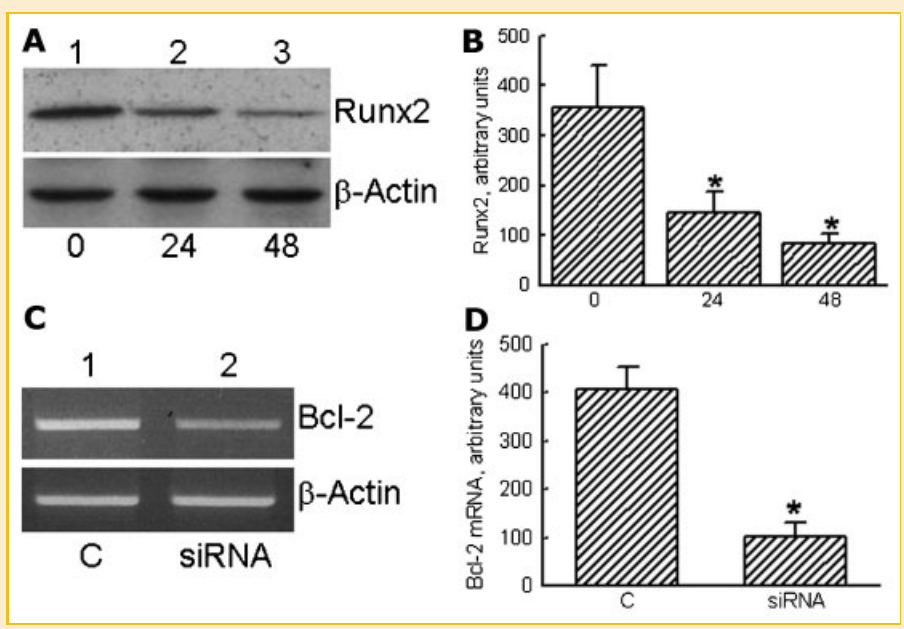

Fig. 6. Effects of Runx2 small interference RNA (siRNA) on production of this transcription factor and Bcl-2 mRNA synthesis. Application of Runx2 siRNA into human MG63 cells for 24 and 48 h. The control cells were treated with scrambled siRNA. Levels of Runx2 and β-actin were immunodetected (A). These protein bands were quantified and analyzed (B). RT-PCR analysis was carried out to determine Bcl-2 mRNA production (C), and these DNA bands were quantified and analyzed (D). Each value represents the mean ± SEM, n = 6. *Values significantly differ from the respective control, $P < 0.05$. C, control.

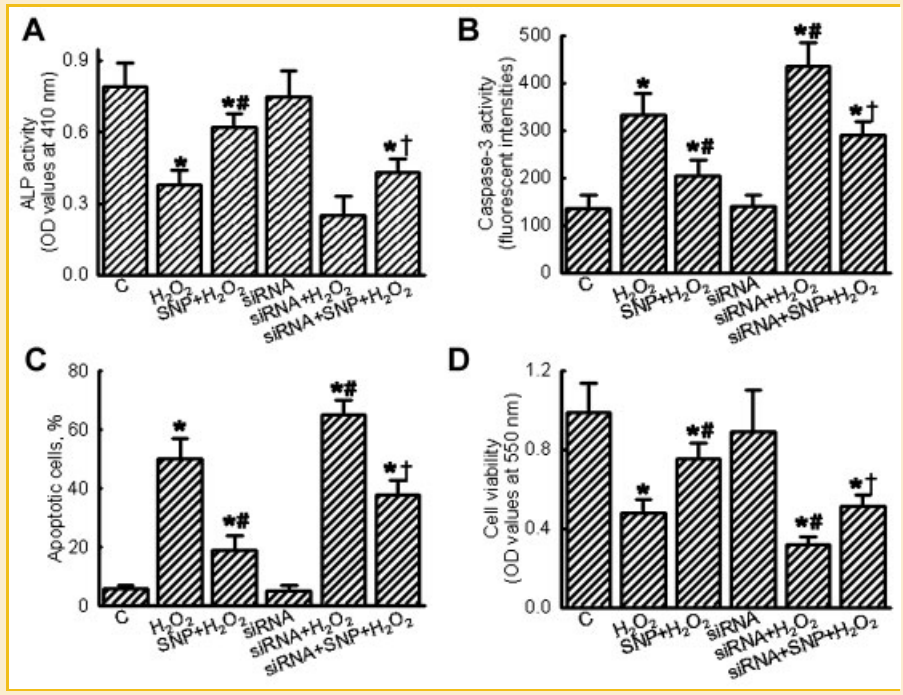

Fig. 7. Effects of Runx2 small interference RNA (siRNA) on protection of pretreatment with 0.3 mM sodium nitroprusside (SNP) against hydrogen peroxide (H_2O_2)-induced osteoblast insults. Runx2 mRNA was knocked-down using RNA interference. Alkaline phosphatase (ALP) activity was analyzed using a colorimetric method (A). Activity of caspase-3 was analyzed using a fluorogenic substrate assay (B). Apoptotic cells were determined by flow cytometry (C). Cell viability was assayed by a colorimetric method (D). Each value represents the mean ± SEM, n = 6. *Values significantly differ from the respective control, and the symbols # and † have respectively statistical signification between cells treated with H_2O_2 and with NO + H_2O_2 as well as with NO + H_2O_2 and with NO + H_2O_2 + siRNA, $P < 0.05$. C, control.

Pretreatment with 0.3 mM SNP did not affect cell viability, but significantly lowered hydrogen peroxide-induced alterations in ALP activity, the mitochondrial membrane potential, caspase-3 activity, cell apoptosis, and cell viability. The apoptotic analysis further revealed that the hydrogen peroxide-induced apoptotic injuries were attenuated by pretreatment with 0.3 mM SNP as an NO donor. Our previous data showed that high NO decreases the mitochondrial membrane potential, complex I NADH dehydrogenase activity, and ATP synthesis, leading to cytochrome *c* release and caspase activation [Chen et al., 2002, 2005b]. The other study executed by our laboratory also showed that pretreatment with 0.3 mM SNP slightly increased the oxidative stress and significantly suppressed high SNP-induced mitochondrial dysfunction and cell death [Chang et al., 2006; Tai et al., 2007]. Thus, the protection of pretreatment with 0.3 mM SNP from hydrogen peroxide-induced osteoblast damage occurs via mitochondria-mediated events rather than due to the reduction of oxidative stress. In cardiomyocytes, hepatocytes, and endothelial cells, NO preconditioning has been shown to have antiapoptotic effects in ischemia and reperfusion injuries [Cottart et al., 1999]. Our present results are also in agreement with previous data found by Figueroa et al. [2005] in cortical neurons. This study provides in vitro data to further show that pretreatment with 0.3 mM SNP as an NO donor can produce antiapoptotic effects on osteoblastic death induced by hydrogen peroxide. During inflammation, reactive oxygen species, including hydrogen peroxide, were massively produced [Symons, 1996; Tai et al., 2007]. The protection of pretreatment with 0.3 mM SNP against hydrogen peroxide-induced insults to osteoblasts may be beneficial to treatment of certain bone diseases such as bone fracture and osteoporosis.

Regulation of *bcl-2* gene expression is involved in the protection of pretreatment with SNP against hydrogen peroxide-induced apoptotic insults to human MG63 cells. Administration of hydrogen peroxide inhibited Bcl-2 mRNA production. In parallel, exposure to hydrogen peroxide significantly decreased the amounts of Bcl-2 in human MG63 cells. Thus, the regulation of Bcl-2 synthesis by hydrogen peroxide occurs possibly via a transcriptional level. Bcl-2 is thought to be an antiapoptotic protein because it can suppress Bax-mediated apoptosis [Luo et al., 1998; Brichese et al., 2004]. A previous study showed that overexpression of the *bcl-2* family genes inhibits Bax translocation and cytochrome *c* release, and prevents cardiac cells from apoptosis [Huang et al., 2005]. A dominant negative form of the Bcl-2 protein produced by deleting the BH1 domain has been reported to be able to trigger cells to undergo apoptosis [Kawatani and Imoto, 2003]. Thus, the recovery in the inhibition of *bcl-2* gene expression in human MG63 cells induced by hydrogen peroxide may be one of the important reasons explaining the protection of 0.3 mM SNP against osteoblast apoptosis. Our unpublished data further showed that both pretreatment with 0.3 mM SNP and exposure to hydrogen peroxide did not affect the levels of Bax mRNA in human MG 63 cells. Therefore, pretreatment with 0.3 mM SNP specifically regulates the expression of *bcl-2* gene.

Runx2 mediates *bcl-2* gene expression, thus plays a critical role in the protection of 0.3 mM SNP as NO donor. Exposure to hydrogen peroxide suppressed the RNA and protein levels of Runx2. The hydrogen peroxide-induced decrease in cellular Runx2 levels may be due to the inhibition of Runx2 mRNA or the enhancement in protein degradation. Pretreatment with 0.3 mM SNP significantly alleviated the suppressive effects. Runx2-DNA binding elements are found in the 5′-end promoter regions of the *bcl-2* gene [Eliseev et al., 2008]. To further validate the role of this transcription factor in the protection of 0.3 mM SNP, Runx2 translation was knocked-down using RNA interference. In parallel with the downregulation of the Runx2 protein, the levels of Bcl-2 mRNA in human MG63 cells were inhibited. Simultaneously, the revered effects of pretreatment with 0.3 mM SNP on hydrogen peroxide-induced suppression of Bcl-2 mRNA synthesis were lowered following administration of Runx2 siRNA. Thus, the Bcl-2 mRNA induction is involved by upregulation of Runx2 protein and RNA levels in human MG63 cells. Consequently, after transfection with Runx2 siRNA, a decrease in *bcl-2* gene expression lowered the protective effects of pretreatment with 0.3 mM SNP on ALP activity, caspase-3 activity, cell apoptosis, and cell viability of human MG63 cells. Runx2 participates in regulation of cell proliferation and differentiation [Eliseev et al., 2008; Hawse et al., 2008]. This is the first study to demonstrate that Runx2 has antiapoptotic effects on the protection of 0.3 mM SNP against oxidative stress-induced osteoblast apoptosis via upregulation of *bcl-2* gene expression. Byon et al. [2008] reported that hydrogen peroxide can increase Runx2 expression and its transactivity. Thus, the effects of hydrogen peroxide on Runx2 activity may be cell-specific.

In conclusion, this study has shown that hydrogen peroxide can induce apoptotic insults to human osteosarcoma MG63 cells through a mitohcondria-caspase protease activation pathway. Pretreatment with 0.3 mM SNP significantly lowered the toxic effects via an anti-apoptotic mechanism. Transcriptional factor Runx2 is involved in upregulation of *bcl-2* gene expression and contributes to the protection of pretreatment with 0.3 mM SNP as an NO donor against hydrogen peroxide-induced osteoblast insults. Therefore, pretreatment with 0.3 mM SNP can protect human MG63 cells from hydrogen peroxide-induced cell apoptosis possibly via Runx2-mediated transcriptional induction of *bcl-2* gene expression. The other study done by our laboratory is using the gain-of-function approach to induce overexpression of *Runx2* gene in human MG63 cells to determine the roles of this transcriptional factor in the protection of pretreatment with 0.3 mM SNP on oxidative stress-induced osteoblast insults.

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