Nitric Oxide Induces Osteoblast Apoptosis through a Mitochondria-Dependent Pathway

WEI-PIN HO,[a] TA-LIANG CHEN,[b] WEN-TA CHIU,[c] YU-TING TAI,[d] AND RUEI-MING CHEN[d,e]

Departments of Orthopedics[a] and Anesthesiology,[d] Wan-Fang Hospital, College of Medicine, Taipei Medical University, Taipei, Taiwan

[b]Taipei City Hospital and Taipei Medical University, Taipei, Taiwan

[c]Division of Neurosurgery, Department of Surgery, Wan-Fang Hospital, College of Medicine, Taipei Medical University, Taipei, Taiwan

[e]Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan

ABSTRACT: Osteoblasts contribute to bone remodeling. Nitric oxide can regulate osteoblast activities. In this study, we attempted to evaluate the pathophysiological effects of nitric oxide on osteoblasts and its possible mechanism using neonatal rat calvarial osteoblasts as the experimental model. Exposure of osteoblasts to sodium nitroprusside, a nitric oxide donor, decreased alkaline phosphatase activities and cell viability in a concentration- and time-dependent manner. Apoptotic analysis revealed that sodium nitroprusside time-dependently increased the percentages of osteoblasts undergoing apoptosis. Administration of sodium nitroprusside reduced the mitochondrial membrane potential of osteoblasts. In parallel with the mitochondrial dysfunction, levels of intracellular reactive oxygen species and cytochrome *c* were significantly elevated following sodium nitroprusside administration. Exposure of osteoblasts to sodium nitroprusside significantly increased caspase-3 activity. Results of this study show that nitric oxide, decomposed from sodium nitroprusside, can induce osteoblast apoptosis through a mitochondrion-dependent cascade that causes mitochondrial dysfunction, release of intracellular reactive oxygen species and cytochrome *c* from mitochondria to cytoplasm, and activation of caspase-3.

KEYWORDS: nitric oxide; osteoblasts; apoptosis; mitochondrial functions; caspase-3

INTRODUCTION

Nitric oxide (NO), synthesized from L-arginine by constitutive or inducible NO synthases, contributes to the regulation of vasodilation, neurotransmission, immuno-

Address for correspondence: Ruei-Ming Chen, Ph.D., Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, No. 250, Wu-Hsing St., Taipei 110, Taiwan. Voice: +886-2-29307930 ext. 2159; fax: +886-2-86621119.
rmchen@tmu.edu.tw; rmchen@wanfang.gov.tw

Ann. N.Y. Acad. Sci. 1042: 460–470 (2005). **© 2005 New York Academy of Sciences.**
doi: 10.1196/annals.1338.039

responses, and death control.[1] Osteoblasts play important roles in bone remodeling.[2] NO has been implicated to be a critical effector to modulate osteoblast activities and bone remodeling.[2,3] Osteoblasts can constitutively produce NO.[4] Following stimulation of inflammatory cytokines, NO will be massively induced by osteoblasts.[5] Constitutive NO can regulate osteoblast proliferation and differentiation.[6,7] However, overproduced NO will damage osteoblasts.[8,9] Our previous study has shown that the NO-induced osteoblast death is mainly via an apoptotic pathway.[10]

A variety of apoptotic factors is involved in programmed cell death.[11,12] Mitochondria are critical energy-producing organelles. Depolarization of mitochondrial membrane potential will lead to release of apoptotic factors, including reactive oxygen species and cytochrome *c*, from mitochondria to cytoplasm.[13,14] These mitochondrial apoptotic factors can sequentially transduce cell death signals. Caspase-3 is one of the cysteine proteases. Activation of caspase-3 will drive cells undergoing apoptosis.[12] However, in osteoblasts, the roles of mitochondrial apoptotic factors and caspase-3 in the NO-induced osteoblast apoptosis are still unknown. This study aimed to evaluate whether NO can modulate mitochondrial function, apoptotic factors, and caspase-3 activity to induce osteoblast apoptosis.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

Osteoblasts were prepared from 3-day-old Wistar rat calvariae following an enzymatic digestion method as described previously.[15] Osteoblasts were seeded in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin and 100 mg/mL streptomycin in tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2.

Sodium nitroprussie (SNP) was purchased from Sigma Corporation (St. Louis, MO). In each independent experiment, SNP was freshly dissolved in phosphate-buffered saline (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) and protected from light. Osteoblasts were treated with various concentrations of SNP for different time intervals.

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.[16] Ten thousand osteoblasts were seeded in 96-well tissue plates culture plates overnight. After incubation with SNP, osteoblasts were cultured with new medium containing 0.5 mg/mL MTT for another 3 h. Blue formazan product in osteoblasts was dissolved in dimethyl sulfoxide (DMSO) and spectrophotometrically measured at a wavelength of 570 nm.

Quantification of Mitochondrial Membrane Potential

Levels of mitochondrial membrane potential were determined according to the method as described previously.[16] In brief, osteoblasts (5×10^5) were seeded in 12-well tissue culture plates overnight and then treated with SNP for different time in-

tervals. After administration of SNP, osteoblasts were harvested and incubated with 3,3′-dihexyloxacarbocyanine ($DiOC_6(3)$), a positively charged dye, at 37°C for 30 min in a humidified atmosphere of 5% CO_2. After washing and centrifuging, cell pellets were suspended with phosphate-buffered saline. Fluorescence intensities in osteoblasts were analyzed by a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA).

Determination of Intracellular ROS

Amounts of intracellular ROS were quantified following the method as described previously.[17] Briefly, osteoblasts (5×10^5) were cultured in 12-well tissue culture plates overnight and then co-treated with SNP and 2′,7′-dichlorofluorescin diacetate, an ROS sensitive dye, for different time intervals. Osteoblasts were harvested and suspended in phosphate-buffered saline. Fluorescence intensities in osteoblasts were quantified by a flow cytometer (FACS Calibur).

Immunoblotting Analysis

Immunoblotting analysis was carried out to determine levels of cytochrome *c* in osteoblasts. After pretreatment with SNP, osteoblasts were washed with phosphate-buffered saline, and cell lysates were collected after dissolving cells in 50 mL of ice-cold radioimmunoprecipitation assay 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 by a bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL). Cytosolic proteins (100 μg) were resolved on 12% polyacrylamide gels and electrophoretically blotted onto nitrocellulose membranes. Cytochrome *c* protein was immunodetected using a mouse monoclonal antibody against rat cytochrome *c* protein (Transduction Laboratories, Lexington, KY). β-actin was immunodetected by a mouse monoclonal antibody against mouse β-actin (Sigma) as an internal control. Intensities of the immunoreactive bands were determined using a UVIDOCMW version 99.03 digital imaging system (Uvtec, Cambridge, England).

Fluorogenic Substrate Assay for Caspase-3 Activity

For determining caspase-3 activity, cell extracts are prepared by lysing osteoblasts in 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 is determined by incubating cell lysate (25 μg total protein) with 50 μm fluorogenic substrate in 200 μL cell-free system buffer comprising 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 were measured by a spectrofluorometer.

Statistical Analysis

Statistical difference between the control and SNP-treated groups was considered significant when the *P* value of the Duncan's multiple range test was less than 0.05.

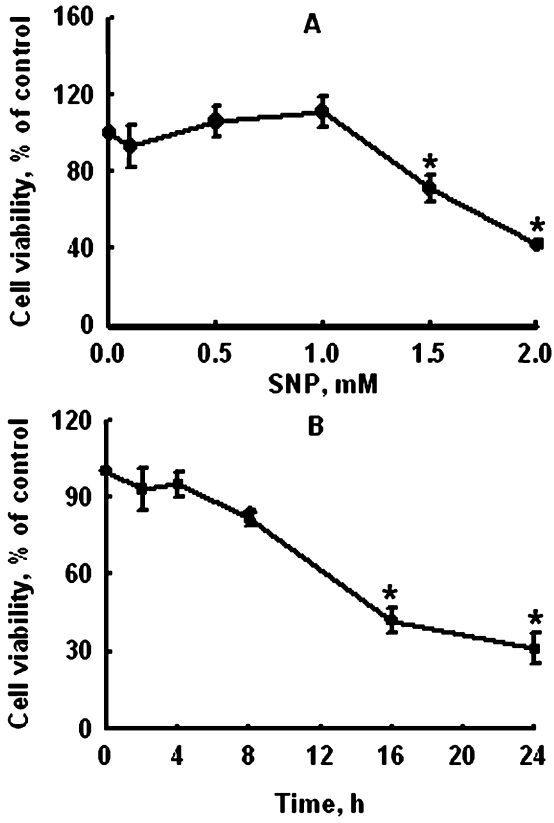

FIGURE 1. Concentration- and time-dependent effects of sodium nitroprusside (SNP) on osteoblast viability. Osteoblasts were exposed to 0.5, 1, 1.5, and 2 mM SNP (**A**) or to 2 mM SNP for 2, 4, 8, 16, and 24 h (**B**). Cell viability was determined by the MTT assay. Each value represents the mean ± SEM for $n = 12$. *Values significantly differ from the respective control, $P < 0.05$.

Statistical analysis between groups over time was carried out by the two-way ANOVA.

RESULTS

Treatment with 0.1, 0.5, and 1 mM SNP for 16 h was not cytotoxic to osteoblasts (FIG. 1A). SNP at 1.5 mM significantly reduced cell viability by 22%. After administration of 2 mM SNP, cell viability was decreased by 58% (FIG. 1A). Exposure to 2 mM SNP for 2 and 4 h did not affect cell viability (FIG. 1B). In 8-h–treated osteoblasts, cell viability was decreased by 19%. Administration of 2 mM SNP in osteoblasts for 16 h significantly decreased cell viability by 59%. Viability of osteoblasts was suppressed by 72% following SNP administration for 24 h (FIG. 1B).

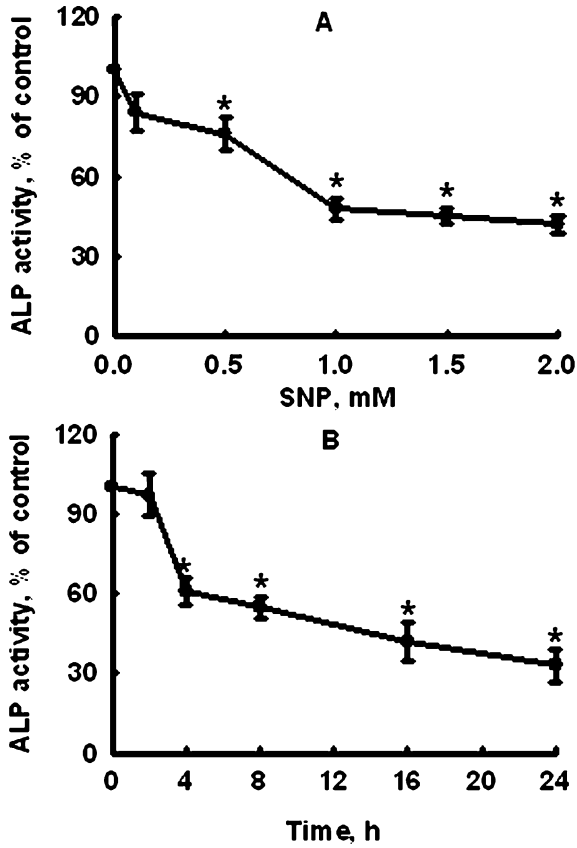

FIGURE 2. Concentration- and time-dependent effects of sodium nitroprusside (SNP) on alkaline phosphatase (ALP) activities. Osteoblasts were exposed to 0.5, 1, 1.5, and 2 mM SNP (**A**) or to 2 mM SNP for 2, 4, 8, 16, and 24 h (**B**). ALP activity was determined by a colorimetric method. Each value represents the mean ± SEM for $n = 12$. *Values significantly differ from the respective control, $P < 0.05$.

Exposure to 0.1 mM SNP for 16 h did not influence alkaline phosphatase (ALP) activity (FIG. 2A). SNP at 0.5 mM significantly decreased ALP activity by 21%. Administration of osteoblasts with 1 and 1.5 mM SNP significantly reduced ALP activity by 49% and 58%, respectively. ALP activity was suppressed by 62% after administration of 2 mM SNP (FIG. 2A). In 2-h–treated osteoblasts, 2 mM SNP did not affect ALP activity (FIG. 2B). After administration of 2 mM SNP in osteoblasts for 4 h, ALP activity was decreased by 39%. Exposure to 2 mM SNP for 8 and 16 h significantly reduced ALP activity by 45% and 59%, respectively. After exposure to 2 mM SNP for 24 h, ALP activity was suppressed by 69% (FIG. 2B).

Analysis by a flow cytometric method revealed that administration of 2 mM SNP for 2 and 4 h did not induce osteoblast apoptosis (FIG. 3). In 8-h–treated osteoblasts, SNP significantly increased apoptotic percentage by 15%. After administration of

TABLE 1. Effects of sodium nitroprusside on the mitochondrial membrane potential and intracellular reactive oxygen species

Mitochondrial membrane potential		Intracellular ROS
Time (h)	(% of control)	(% of control)
0	100	100
1	98 ± 6	181 ± 33*
2	82 ± 5*	303 ± 85*
4	62 ± 8*	528 ± 74*

NOTE: Osteoblasts were exposed to 2 mM sodium nitroprusside for 1, 2, and 4 h. Levels of mitochondrial membrane potential and intracellular reactive oxygen species (ROS) were determined by a flow cytometer. Each value represents the mean ± SEM for $n = 6$. *Values significantly differ from the respective control, $P < 0.05$.

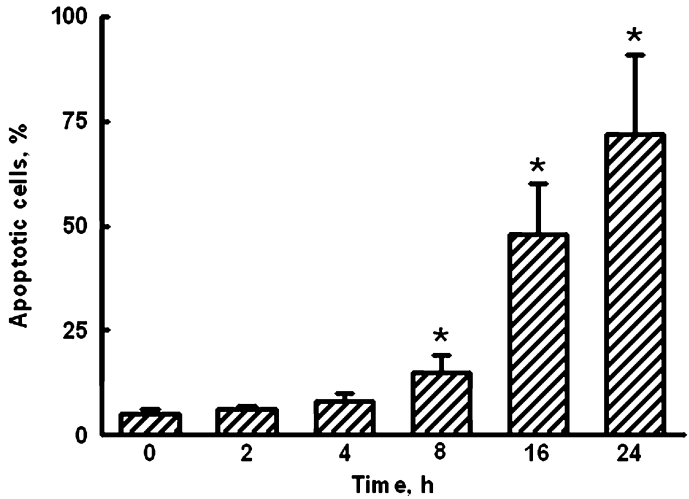

FIGURE 3. Time-dependent effects of sodium nitroprusside (SNP) on osteoblast apoptosis. Osteoblasts were exposed to 2 mM SNP for 2, 4, 8, 16, and 24 h. Apoptotic cells were determined by a flow cytometric method. Each value represents the mean ± SEM for $n = 9$. * Values significantly differ from the respective control, $P < 0.05$.

SNP for 16 h, almost 50% of osteoblasts underwent apoptosis. In 24-h–treated osteoblasts, SNP increased 68% of osteoblasts undergoing apoptosis (FIG. 3).

In 1-h–treated osteoblasts, SNP did not affect mitochondrial membrane potential (TABLE 1). Administration of osteoblasts with SNP for 2 h significantly decreased mitochondrial membrane potential by 18%. After administration of SNP for 4 h, the mitochondrial membrane potential was reduced by 38%. Exposure to SNP for 1 h significantly increased levels of intracellular ROS by 81% (TABLE 1). After administration of SNP for 2 h, levels of intracellular ROS were augmented by 203%. In 4-h–treated osteoblasts, SNP significantly enhanced levels of intracellular ROS by 428% (TABLE 1).

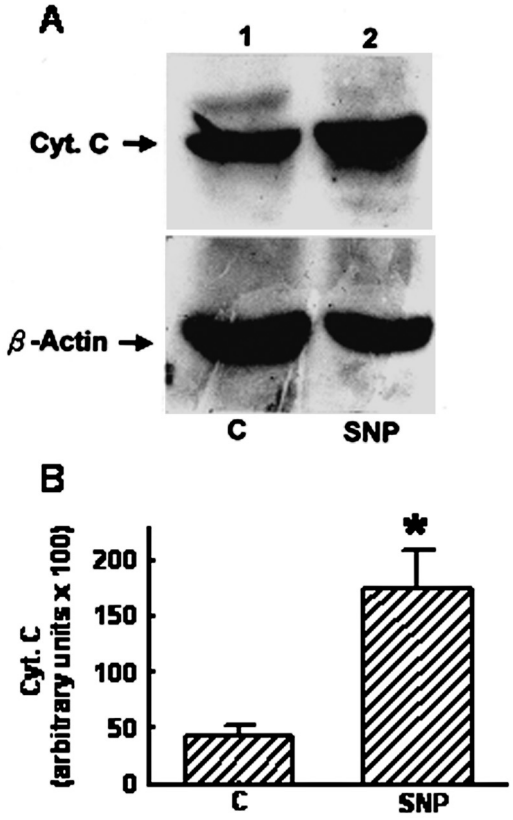

FIGURE 4. Effect of sodium nitroprusside (SNP) on cytochrome *c* (Cyt. C) production. Osteoblasts were exposed to 2 mM SNP for 8 h. Cytochome *c* in osteoblasts was immunodetected using a monoclonal antibody against rat cytochrome *c* protein (**A**). β-actin was immunodetected to be an internal control. Intensities of immunorelated protein bands were quantified by a digital system (**B**). Each value represents the mean ± SEM for $n = 4$. *Values significantly differ from the respective control, $P < 0.05$.

Immunoblotting analysis revealed that cytochrome *c* was detectable in untreated osteoblasts (FIG. 4A, lane 1). After administration of osteoblasts with SNP for 8 h, amounts of cytochrome *c* protein were significantly enhanced (lane 2). Intensities of immunorelated protein bands were quantified by a digital imaging system, and data are shown in FIGURE 4B. Exposure to SNP significantly increased amounts of cytochrome *c* protein by 3.4-fold.

Activity of caspase-3 was determined by a fluorogenic substrate assay. In untreated osteoblasts, low levels of caspase-3 activity were detected (FIG. 5). Administration of osteoblasts with SNP significantly increased caspase-3 activity by 5.5-fold.

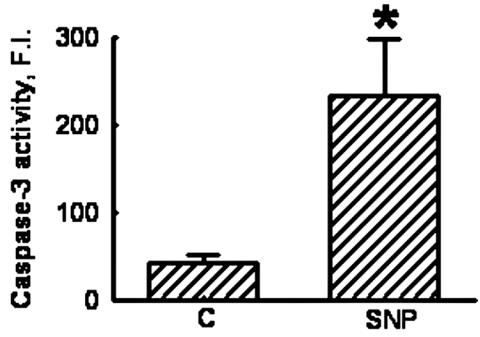

FIGURE 5. Effect of sodium nitroprusside (SNP) on caspase-3 activity. Osteoblasts were exposed to 2 mM SNP for 8 h. Caspase-3 activity was determined by a fluorogenic substrate assay. Each value represents the mean ± SEM for $n = 6$. *Values significantly differ from the respective control, $P < 0.05$.

DISCUSSION

SNP significantly decreased osteoblast viability in a concentration- and time-dependent manner. SNP can be decomposed to NO under light exposure or a biological reducing system.[18] Analysis by the Griess reaction method revealed that administration of osteoblasts with SNP concentration- and time-dependently increased nitrite production (data not shown). Nitrite is one of oxidative metabolites of NO.[1] Elevation of nitrite corresponds to the increase in NO levels. ALP is a mark protein synthesized in osteoblasts.[2] This study showed that SNP significantly decreased ALP activity. Thus, data from the present study demonstrates that NO decomposed from SNP can induce osteoblast insults, even death. Apoptotic analysis revealed that SNP time-dependently increased the percentage of osteoblasts undergoing apoptosis. Thus, the SNP-induced osteoblast death is mainly through an apoptotic pathway. Our preliminary study used a specific inhibitor of cyanide to co-treat osteoblasts with SNP; and the data revealed that this inhibitor could not block SNP-caused osteoblast insults (data not shown). In addition, administration of GS-NO, another NO donor, in osteoblasts could also induce cell apoptosis. Therefore, NO, rather than cyanide, plays a major role in SNP-induced osteoblast apoptosis.

SNP time-dependently decreased mitochondrial membrane potential. Maintenance of the mitochondrial membrane potential is critical to the synthesis of cellular adenosine triphosphate.[19] Depletion of cellular adenosine triphosphate will lead to cell dysfunction or apoptosis. Thus, one of possible mechanisms to explain the SNP-induced osteoblast apoptosis may be via the suppression of the mitochondrial membrane potential and cellular adenosine phosphate synthesis by this NO donor. In addition, depolarization of the mitochondrial membrane potential has been reported to increase the release of apoptotic factors from mitochondria to cytoplasm.[14] Enhancement of cytosolic apoptotic factors can drive cells to apoptosis. This study shows that SNP increased levels of intracellular ROS and cytochrome *c* in osteoblasts. ROS and cytochrome *c* are two typical apoptotical factors.[13,14] Therefore, the

other mechanism to explain the SNP-induced osteoblast apoptosis may be through the sequentially modulating effects of this NO donor on the mitochondrial membrane potential and apoptotic factor release.

Administration of osteoblasts with SNP increased levels of intracellular ROS time-dependently. In mitochondria, ROS is a by-product synthesized during the respiratory chain reaction. Disruption of the mitochondrial membrane potential will lead to the release of ROS from mitochondria to cytoplasm.[13,14] Thus, one of the possible sources for the SNP-enhanced intracellular ROS production in osteoblasts is due to the release of ROS from mitochondria. This study used DCFH-DA dye to catch ROS. A previous study showed that NO and peroxynitrite, an oxidative product of NO and superoxide, can directly react with DCFH-DA.[20] Thus, NO decomposed from SNP may be another source of ROS in osteoblasts after exposure to this donor. Elevation of ROS will increase cellular oxidative stress. Cumulative evidence suggests that ROS can trigger cell apoptosis by upregulating pro-apoptotic Bax protein.[21] Our previous study showed that NO can increase *de novo* synthesis of Bax protein in osteoblasts.[10] Therefore, the SNP-induced ROS is involved in osteoblast apoptosis.

SNP increased cytochrome *c* release from mitochondria to cytoplasm. Depolization of the mitochondrial membrane potential can increase cytochrome *c* release.[13,14] Several lines of evidence show that Bcl-2 family members, including Bax and Bak, can bind to mitochondrial membrane and promote cytochrome *c* release.[22] Thus, NO decomposed from SNP may modulate mitochondrial function and Bcl-2 family production to enhance the release of cytochrome *c* from mitochondria. Another previous study showed that cytochrome *c* release will result in caspase activation and cell apoptosis.[23] Therefore, the SNP-induced release of cytochrome *c* is involved in osteoblast apoptosis.

Caspase-3 activity was augmented following SNP administration. A variety of apoptotic factors contribute to the activation of capase-3. This study demonstrated that SNP significantly increased intracellular ROS in osteoblasts. ROS has been reported to be capable of activating caspase-3.[19] Release of cytochrome *c* was enhanced following SNP administration. After binding to the adaptor Apaf-1, cytochrome *c* can activate procaspase-9 to mature caspase-9.[23] Sequential cleavage of procaspase-3 by caspase-9 will activate caspase-3. Thus, an increase in capase-3 activity following SNP administration may derive from enhancement of intracellular ROS and cytochrome *c* release. Active caspase-3 plays an important role in cell death control.[12] Caspase-3 can degrade key proteins in cells and drive cells undergoing apoptosis. Therefore, activation of caspase-3 plays a critical role in SNP-induced osteoblast apoptosis.

CONCLUSION

This study has shown that SNP increases levels of NO in osteoblasts, and decreases ALP activity and cell viability. The SNP-induced osteoblast death is mainly via an apoptotic pathway. SNP can reduce the mitochondrial membrane potential. In parallel to mitochondrial dysfunction, SNP enhances release of intracellular ROS and cytochrome *c* from mitochondria to cytoplasm. Following an increase in levels of mitochondrial apoptotic factors, caspase-3 activity is augmented after SNP administration. According to the present data, we suggest NO decomposed from SNP

can induce osteoblast apoptosis via the sequential modulation of mitochondrial function, release of mitochondrial apoptotic factors, and caspase-3 activity.

ACKNOWLEDGMENTS

This study was supported by Grant 93TMU-WFH-07 from the Wang-Fang Hospital and Taipei Medical University, Taipei, Taiwan.

REFERENCES

1. MONCADA, S., R.M. PALMER & E.A. HIGGS. 1991. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. **43:** 109–142.
2. COLLIN-OSDOBY, P., G.A. NICKOLS & P. OSDOBY. 1995. Bone cell function, regulation, and communication: a role for nitric oxide. J. Cell. Biochem. **57:** 399–408.
3. EVANS, D.M. & S.H. RALSTON. 1996. Nitric oxide and bone. J. Bone Miner. Res. **11:** 300–305.
4. HEFLICH, M.H., D.E. EVANS, P.S. GRABOWSKI, *et al*. 1997. Expression of nitric oxide synthase isoforms in bone and bone cell cultures. J. Bone Miner. Res. **12:** 1108–1115.
5. DAMOULIS, P.D. & P.V. HAUSCHKA. 1994. Cytokines induce nitric oxide production in mouse osteoblasts. Biochem. Biophys. Res. Commun. **201:** 924–931.
6. CHAE, H.J., R.K. PARK, H.T. CHUNG, *et al*. 1997. Nitric oxide is a regulator of bone remodelling. J. Pharm. Pharmacol. **49:** 897–902.
7. HIKIJI, H., W.S. SHIN, S. OIDA, *et al*. 1997. Direct action of nitric oxide on osteoblastic differentiation. FEBS Lett. **410:** 238–242.
8. DAMOULIS, P.D. & P.V. HAUSCHKA. 1997. Nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. J. Bone Miner. Res. **12:** 412–422.
9. MOGI, M., K. KINPARA, A. KONDO, *et al.* 1999. Involvement of nitric oxide and biopterin in proinflammatory cytokine-induced apoptotic cell death in mouse osteoblastic cell line MC3T3-E1. Biochem. Pharmacol. **58:** 649–654.
10. CHEN, R.M., H.C. LIU, Y.L. LIN, *et al*. 2002. Nitric oxide induces osteoblast apoptosis through the de novo synthesis of Bax protein. J. Orthop. Res. **20:** 295–302.
11. CHUNG, K.C., J.H. PARK, C.H. KIM, *et al*. 1999. Tumor necrosis factor-alpha and phorbol 12-myristate 13-acetate differentially modulate cytotoxic effect of nitric oxide generated by serum deprivation in neuronal PC12 cells. J. Neurochem. **72:** 1482–1488.
12. GOYAL, L. 2001. Cell death inhibition: keeping caspases in check. Cell **104:** 805–808.
13. HORTELANO, S., A.M. ALVAREZ & L. BOSCA. 1999. Nitric oxide induces tyrosine nitration and release of cytochrome *c* preceding an increase of mitochondrial transmembrane potential in macrophages. FASEB J. **13:** 2311–2317.
14. BLOM, W.M., H.J. DE BONT & J.F. NAGELKERKE. 2003. Regional loss of the mitochondrial membrane potential in the hepatocyte is rapidly followed by externalization of phosphatidylserines at that specific site during apoptosis. J. Biol. Chem. **278:** 12467–12474.
15. PARTRIDGE, N.C., D. ALCORN, V.P. MICHELANGELI, *et al*. 1981. Functional properties of hormonally responsive cultured normal and malignant rat osteoblastic cells. Endocrinology **108:** 213–219.
16. WU, C.H., T.L. CHEN, T.G. CHEN, *et al*. 2003. Nitric oxide modulates pro- and anti-inflammatory cytokines in lipopolysaccharide-activated macrophages. J. Trauma **55:** 540–545.
17. CHEN, R.M., C.H. WU, H.C. CHANG, *et al*. 2003. Propofol suppresses macrophage functions and modulates mitochondrial membrane potential and cellular adenosine triphosphate levels. Anesthesiology **98:** 1178–1185.

18. KOWALUK, E.A., P. SETH & H.L. FUNG. 1992. Metabolic activation of sodium nitroprusside to nitric oxide in vascular smooth muscle. J. Pharmacol. Exp. Ther. **262:** 916–922.
19. YU, X.H., T.D. PERDUE, Y.M. HEIMER, *et al*. 2002. Mitochondrial involvement in tracheary element programmed cell death. Cell Death Differ. **9:** 189–198.
20. RAO, K.M., J. PADMANABHAN, D.L. KILBY, *et al.* 1992. Flow cytometric analysis of nitric oxide production in human neutrophils using dichlorofluorescein diacetate in the presence of a calmodulin inhibitor. J. Leukoc. Biol. **51:** 496–500.
21. KUMAR, D. & B.I. JUGDUTT. 2003. Apoptosis and oxidants in the heart. J. Lab. Clin. Med. **142:** 288–297.
22. SCORRANO L. & S.J. KORSMEYER. 2003. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem. Biophys. Res. Commun. **304:** 437–444.
23. BORUTAITE, V. & G.C. BROWN. 2003. Mitochondria in apoptosis of ischemic heart. FEBS Lett. **541:** 1–5.