行政院國家科學委員會專題研究計畫成果報告

粒線體在一氧化氮誘導骨母細胞凋零所扮演的角色研究(1/2)

STUDY OF ROLE OF MITOCHONDRIA ON NITRIC OXIDE-INDUCED OSTEOBLAST APOPTOSIS

計畫類別：v 個別型計畫　　□整合型計畫

計畫編號：NSC 90－2314－B－002－196－

執行期間：2001 年 08 月 01 日至 2002 年 07 月 31 日

計畫主持人：劉華昌

共同主持人：陳瑞明

本成果報告包括以下應繳交之附件：

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：台大醫學院骨科

台北醫學大學醫學系

中　華　民　國九十一年八　月　十　日

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計畫編號：NSC 90-2314-B-002-196
執行期限：90 年 08 月 01 日至 91 年 07 月 31 日
主持人：劉華昌　台大醫學院骨科
共同主持人：陳瑞明　台北醫學大學醫學系
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Abstract

In the first-year study of our project, we demonstrated that NO, which was released from sodium nitroprusside, altered osteoblast morphologies, decreased alkaline phosphatase activities and cell viability. NO also increased the percentage of apoptotic cells in osteoblasts and caused nuclei DNA fragmentation. In parallel to osteoblast dysfunction, NO increased the levels of cellular reactive oxygen species and reduced mitochondrial membrane potential. Following administration of sodium nitroprusside, the intracellular calcium was elevated. From the present data, we suggest mitochondria might have a critical role in NO-induced osteoblast apoptosis.

Keywords: Osteoblast, Nitric oxide, Apoptosis, Mitochondria, Reactive oxygen species, Mitochondrial membrane potential, Intracellular calcium

Introduction

Bone remodeling is characterized by the coupling of osteoblast-mediated bone formation and osteoclast-mediated bone resorption. A complicated network of systemic and local signals including vitamin D metabolites, PTH, and local growth factors or cytokines such as IGF-1, TGF-β, and bone morphogenetic proteins regulates osteoblast-mediated bone formation (Collin-Osdoby et al., 1995). Osteoblasts produce varieties of oxidants including nitric oxide and superoxides. It is possible that ROS may also participate in modulating osteoblast-mediated bone formation.

Nitric oxide (NO) is produced from L-arginine by nitric oxide synthase (NOS) and is involved in various pathophysiological processes in many tissues (Moncada et al., 1991). Recent works suggest that NO play an important role as a paracrine and autocrine mediator of bone cell activity in response to diverse stimuli, such as cytokine activation, sex hormone deficiency and mechanical strain (Riancho et al., 1995). Extravascular NO can be produced by osteoblasts (Anbar and Gratt, 1998). Some of the cytotoxic effects of NO may be mediated by inhibition of mitochondroal respiration. NO can rapidly, potently, and reversibly inhibit mitochondrial respiration at respiratory complex Ⅳ (cytochrome oxidase), whereas peroxynitrate ($ONOO^-$,formed in the reaction between NO and superoxide) can inhibit mitochondrial aconitase, complexes Ⅰ, Ⅱ, and Ⅴ, as well as opening the mitochondrial permeability transition pore (mPTP)(Browm, 1999).

Our previous study (NSC88-2314-B002-256) had demonstrated that high concentration of NO caused osteoblast apoptosise. We suggest that high concentration of NO is cytotoxic to rat ostetoblasts, and the cell death mechanism is

through Bax protein-involved apoptotic process. This project is further designed to study the role of mitochondria in NO-induced osteoblast apoptosis. The first year of this project is aimed to investigate 1) the effect of NO on mitochondrial membrane potential of rat osteoblasts; 2) the effect of NO on the level of intracellular ROS; 3) the effect of NO on the level of intracellular calcium concentration.

Materials and Methods

Rat osteoblasts were prepared from 3-day-old Wistar rat calvarias following the sequentially enzymatic digestion method as described previously (Partridge et al, 1981). The primary osteoblasts were maintained in Dulbecco's modified Eagle medium (Gibco, BRL, Grand Island, NY, USA), supplemented with a 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin in 250 ml polystyrene tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2. WEGSB was dissolved in dimethylsulfoxide (DMSO). To rule out the toxicity of DMSO, the concentration of the solvent was less than 0.1% (vol./vol.).

Osteoblast viability was determined by testing the mitochondrial enzyme function according to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described previously (Carmichael et al., 1987). Briefly, ten thousand rat osteoblasts were sub-cultured into a 96-well tissue culture cluster overnight. After WEGSB treatment, osteoblasts were washed with 1 X PBS (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). The medium was renewed with a nutrient DMEM medium containing 0.5 mg/ml MTT, and cells were cultured for another 3 hours. After adding DMSO, the cell lysates were colorimetrically detected using an ELISA reader (MRX_{TC}, Dynex Technology, Chantilly, VA, USA) at a wavelength of 570 nm.

Alkaline phosphatase activity was analyzed according to the colorimetric protocol provided in the Sigma Diagnostics Alkaline, Acid and Prostatic Acid Phosphotase kit (Sigma Diagnostics, Inc, St. Louis, MO, USA). Briefly, ten thousand rat osteoblasts were sub-cultured in a 96-well tissue culture cluster overnight. After treatment, the extraction buffer (0.05% Triton X-100, 2 mM $MgCl_2$) was added to osteoblasts, and the extracts were used for alkaline phosphatase activity assay using *p*-nitrophenyl phosphate as a substrate to detect the formation of yellow hydrolytic product, *p*-nitrophenol, using an ELISA reader at a wavelength of 410 nm.

The Klenow labeling procedure was used as described. Cells were harvested by a 5-minute centrifugation at 2000 rpm and resuspended in 10 mmol/L Tris, 1 mmol/L EDTA, and 0.5% Triton X-100, pH 8.0 (10^6 cells per 50μl of buffer). Extracts were incubated on ice for 20 minutes and briefly vortexed every 5 minutes. Cell debris and high molecular weight DNA were removed by a 10-minute centrifugation at 14,000 rpm at 4°C. Supernatants were extracted with phenol and phenol/chloroform and precipitated with 0.5 volumes of 7.5 mmol/L ammonium acetate and 2 volumes of ethanol for 2 hours at -80°C. DNA was resuspended in TE (10 mmol/L Tris-Cl, pH 7.4, and 1 mmol/L EDTA, pH8.0) and 1 μg was treated with 5 U of Klenow polymerase (Boehringer Mannheim Indianapolis, IN) and 5μCi of {^{32}P}dCTP for 10 minutes in the presence of 10 mmol/L Tris, pH 7.5, and 1 mmol $MgCl_2$ as described. Unincorporated nucleotides were removed by three consecutive precipitations. One-half of the labeled DNA was separated on 2% agarose gels, dried, and exposed to X-ray film.

Intracellular ROS of rat osteoblasts were determined using the flow cytometric method as described previously (Simizu et al., 1997). Osteoblasts (5 x 10^5) were sub-cultured in a 12-well tissue culture cluster overnight and then treated with WEGSB for 4 hours. An ROS-sensitive dye, 2',7'-dichlorofluorescin diacetate, was co-cultured with WEGSB to catch peroxide radicals in rat osteoblasts. The relative fluorescence intensity in cells was quantified by a flow cytometer.

The membrane potential of osteoblastic mitochondria was determined using the flow

cytometric method as described previously [Chen, 1988]. Osteoblasts were sub-cultured into a 12-well tissue culture cluster overnight and then treated with WEPCC and fluorescent dye 3,3'-dihexyloxacarbocyanine iodide for 4 hours. The relative fluorescence intensity in cells was quantified by a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA, USA).

In order to validate the effect of NO on the alteration of intracellular calcium concentration, we used osteoblasts to measure the calcium immobilization within the cells pre-incubated with or without SNP. Using Fluo-3 staining and a fluorescence spectrophotometer (confocal microscope), intracellular calcium immobilization was demonstrated by the appearance of "hot spots" within the cytoplasm and perinuclear regions after addition of bradykinin to the cells. The changes of fluorescence density measured within these areas versus the effect of time were analyzed and compared with the cells in control group.

Results

Exposure of osteoblasts to 2 mM SNP for 16 hours increased 5.5-fold of nitrite (Table 1). SNP caused 41 and 49 % decreases in ALP activity and cell viability, respectively. The percentage of macrophages undergoing apoptosis acchived 60 % followin administration of SNP.

Osteoblast morphologies were observed and photographed following treatments of 2 mM SNP for 2, 4, 8, 16 and 24h. In the 4h-treated group, some osteoblasts' morphologies were altered by SNP (Fig. 1). In the 8h-treated group, rough rounded and floating osteoblasts were markedly observed. In the 16h-treated group, 50% osteoblasts became shrunk, rounded and floated. In the 24h-treated group, almost 80% of the osteoblasts were detached and floating.

Genomic DNA from the control and SNP-treated osteoblasts were isolated and electrophoretically separated in agarose gel (Fig. 2). Administration of osteoblasts with SNP caused 100 base-pair fragments of the DNA ladder, but the effect was not observed in untreated osteoblasts (lanes 2 and 3).

Exposure of osteoblasts to 2 mM SNP for 1, 2 and 4 hours resulted in 70 %, 3-and5-fold increases in the levels of intracellular reactive oxygen species (Fig. 3).

In 1 hour-treated osteoblasts, PPF did not affect the membrane potential of mitochomdria (Fig. 4). However, when treating with PPF for 2 and 4 hours, the membrane potential was decreased by 15 and 28 %, respectively.

Exposure of macrophages to SNP for 30 min led to an increase in intracellular calcium (Fig. 5). However, the SNP-caused increase was descended after one hour.

Conclusion

The first-year study of our project has shown that NO, released from SNP, can alternate osteoblast morphologies, decrease ALP activity and cell viability, increase the percentage of apoptotic cells, and causes DNA fragmentation. SNP promoted the levels of cellular reactive oxygen species and decreased mitochondrial membrane potential. Administration of SNP elevated intracellular calcium distribution. Mitochondria could contribute to the SNP-induced effects in osteoblasts. Therefore, mitochondria might play an important role in NO-induced osteoblast apoptosis.

Further Study

The 2nd year project is designed to study 1) the effect of NO on cytochrome c release from mitochondria; 2) the effect of NO on the regulation of Bcl-2 family proteins; 3) the effect of NO on the regulation of Bax protein; 4) the effect of NO on the activations of caspases; 5) In addition, the correlation between gene expression (cpp32 and hsp70) of apoptosis will also be investigated.

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Simizu S, Imoto M, Masuda N, Takada M, Umezawa K: Involvement of hydrogen peroxide production in erbstain-induced apoptosis in human small cell lung carcinoma cells. Cancer Res 1997; 56: 4978-82.

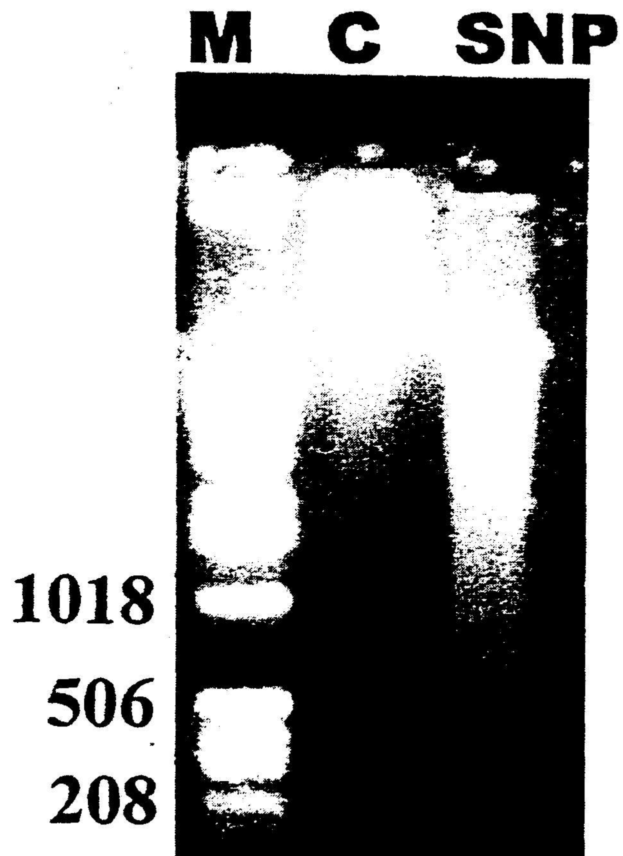

Fig. 2 SNP-induced DNA ladder in osteoblasts. Osteoblasts were treated with 2 mM SNP for 16h. Genomic DNA from control (C) and SNP-treated osteoblasts were isolated and electrophoretically separated in 1.2% agarose gel containing 0.1 mg/ml ethedium bromide. M: DNA marker.

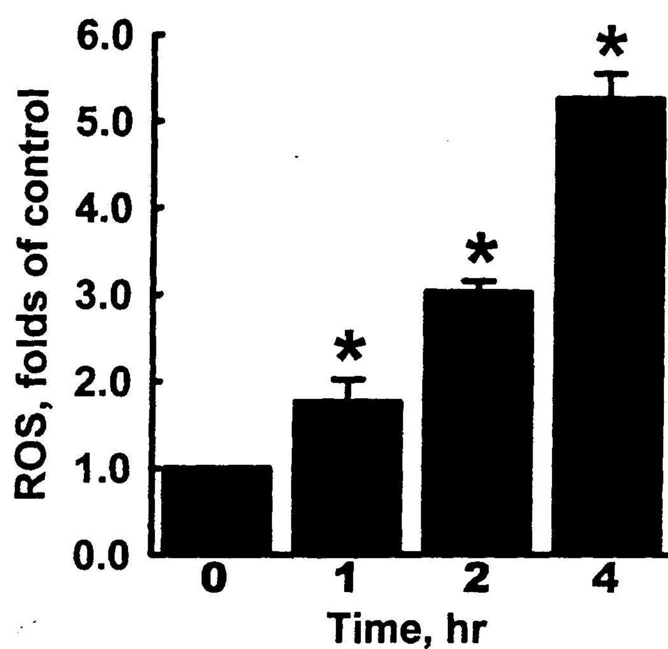

Fig. 3 Time-dependent effects of SNP on increase of intracellular ROS in rat osteoblasts. * Value significantly different from the respective control, $P < 0.05$.

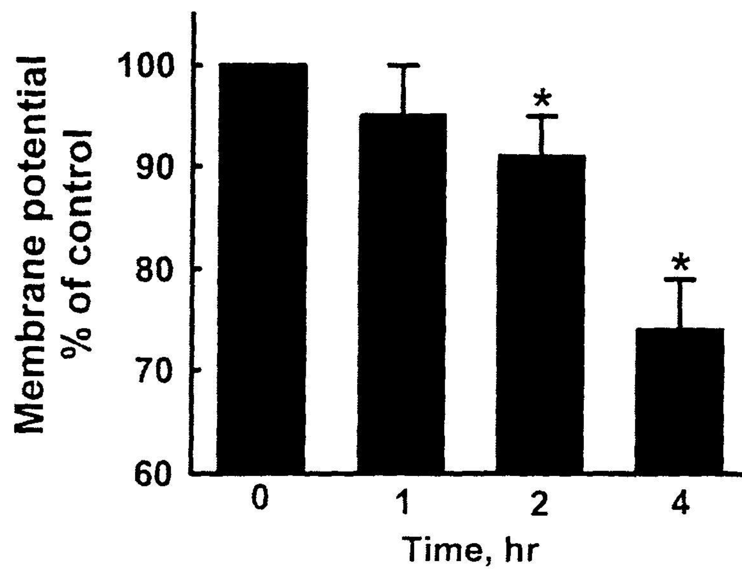

Fig. 4 Time-dependent effects of SNP on decrease of mitochondrial membrane potential in rat osteoblasts. * Value significantly different from the respective control, $P < 0.05$.

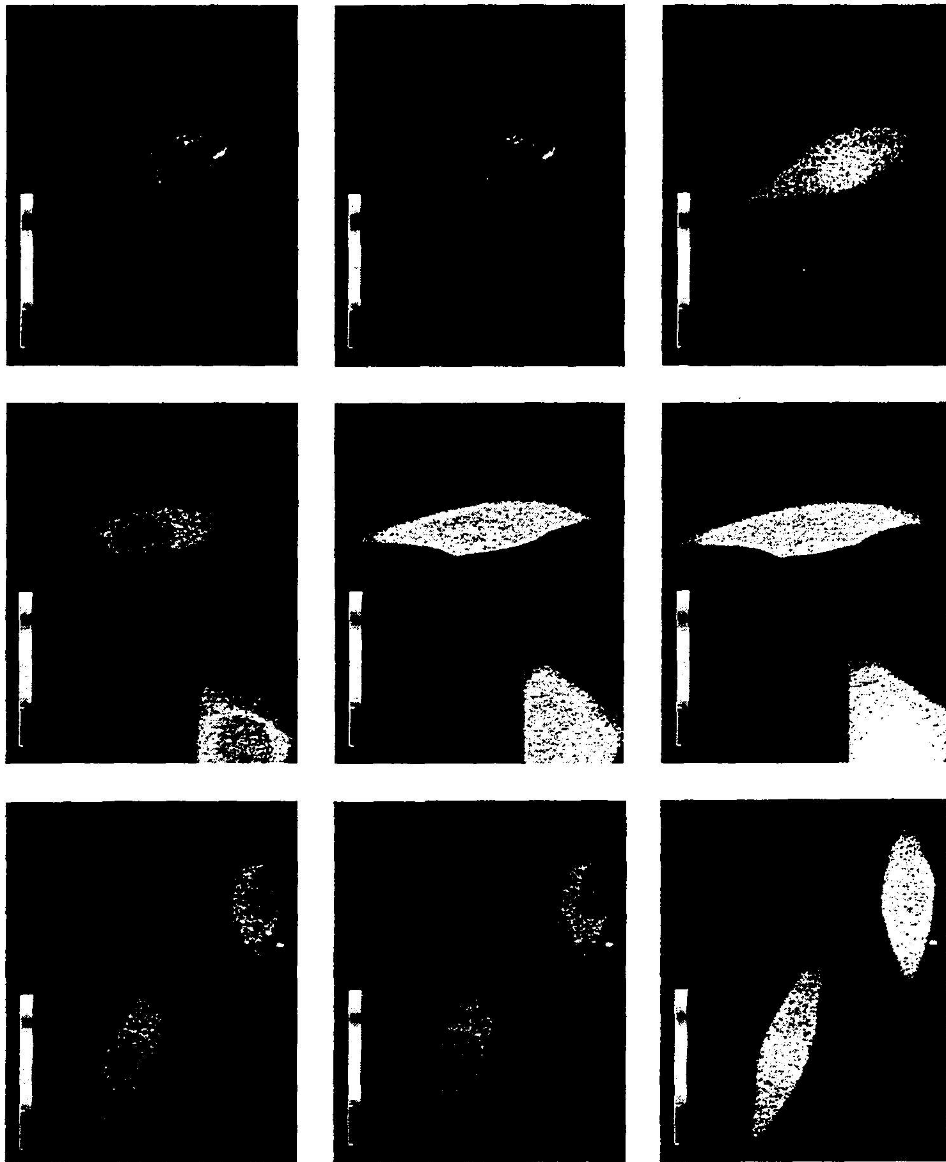

Fig. 5 Effects of SNP on bradykinin-caused intracelluar Ca^{2+} release in osteoblasts incubated with Fluo-3 under a confocal microscope. Osteoblasts were treated with 2 mM SNP for 0 (*top three panels*), 30 (*middle three panels*) and 60 min (*bottom three panels*), and the cells were incubated with Fluo-3 dye for 1 hr. The distribution of intracellular Ca^{2+} was monitored following the bradykinin treatment for 0 (*left three panels*), 10 (*middle three panels*) and 30 sec (*right three panels*).

九十年度 【粒線體在一氧化氮誘導骨母細胞凋零所扮演的角色研究(1/2)】經費核定清單

執行機關：國立台灣大學醫學院骨科　　　主　持　人：劉華昌　　教授

補助項目	申請金額	核定金額	說　明
人事費	409,500	408,010	1. 學士級專任助理第一年1名，月支29,700元(12.625月計) 2. 雇主負擔之勞健保費共33,048元(90.08.01 起始列計) (學士級專任助理第一年 2,754元 x 12.000月 x 1名) 3. 勞健保費用、年終工作獎金部份均不得流用
研究設備費	90,000	45,000	1. Sigma Plot等資訊軟體 45,000元
其他費用	823,100	318,870	1. 大白鼠，飼料費，動物及寄養費等實驗動物 35,000元 2. 細胞培養製劑，各類塑膠材料，組織培養皿，螢光標記反應劑，單株抗體，玻璃器材，X光底片等消耗性器材 256,370元 3. 電腦使用費 10,000元 4. 文具紙張，郵電費，電腦耗材，影印費等雜支 10,000元 5. 論文發表費 7,500元
管理費	79,356	61,120	1. 90.08.01起執行之計畫調整為8%(研究設備費最高以10萬元計)
合　計	1,401,956	833,000	執行期限： 90/08/01 ～ 91/07/31 計畫編號： NSC 90-2314-B-002 -196 -

研究類型：一般型研究計畫(個別型)　　多年期計畫　學門名稱： 骨科　　流水號： 90WFA0100261
研究性質：基礎研究　　承辦人： 蔡馥芬
應繳報告：期中報告(請於計畫執行期滿二個月前，併同下年度經費預核清單送本會，以憑核定下年度經費)
研究成果歸屬：國立台灣大學

行政院
國家科學委員會

九十一年度 【 粒線體在一氧化氮誘導骨母細胞凋零所扮演的角色研究(2/2) 】經費預核清單

執行機關：國立台灣大學醫學院骨科

主 持 人：劉華昌 教授

補助項目	申請金額	核定金額	說明
人事費	409,500	455,880	1. 學士級專任助理第三年1名，月支31,200元(13.500月計) 2. 雇主負擔之勞健保費共34,680元(90.08.01 起始列計) (學士級專任助理第三年 2,890元 x 12.000月 x 1名) 3. 勞健保費用、年終工作獎金部份均不得流用
研究設備費	100,000	45,000	1. 電泳槽，轉置槽，電源供應器等儀器設備 45,000元
其他費用	844,100	252,500	1. 大白鼠，飼料費，動物及寄養費等實驗動物 35,000元 2. 細胞培養製劑，各類塑膠材料，組織培養皿，螢光標記反應劑，單株抗體，玻璃器材，X光底片等消耗性器材 190,000元 3. 電腦使用費 10,000元 4. 文具紙張，郵電費，電腦耗材，影印費等雜支 10,000元 5. 論文發表費 7,500元
管理費	81,216	60,220	1. 90.08.01起執行之計畫調整為8%(研究設備費最高以10萬元計)
合　　計	1,434,816	813,600	執行期限： 91/08/01 ～ 92/07/31 計畫編號： NSC 91-2314-B-002 -067 -

研究類型：一般型研究計畫(個別型)
研究性質：基礎研究
應繳報告：精簡報告
研究成果歸屬：國立台灣大學

多年期計畫 學門名稱： 骨科

流水號： 91PFA0100261
承辦人： 蔡葭芬

行 政 院
國家科學委員會
綜合業務處(二)科