行政院國家科學委員會補助專題研究計畫成果報告

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※ **一氧化氮對大鼠骨母細胞抗細胞凋零作用之研究** ※
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執行期間：89 **年** 08 **月** 01 **日至** 90 **年** 07 **月** 31 **日**

計畫主持人：劉華昌

共同主持人：陳瑞明

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行政院國家科學委員會專題研究計畫成果報告
一氧化氮對大鼠骨母細胞抗細胞凋零作用之研究
Study of Antiapoptotic Effects of Nitric Oxide on Rat Osteoblasts

計畫編號：NSC　89－2314－B－002－396－
執行期限：89 年 08 月 01 日至 90 年 07 月 31 日

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一、中文摘要

國科會自八十七年度起補助專題計畫成果報告準備方式有所變革，本文提供一個統一格式[*]，可供主持人撰寫報告時參考使用。

關鍵詞：專題計畫、報告格式、國科會

Abstract

Nitric oxide (NO) is an important mediator involved in the osteoblast- and osteoclast-mediated bone remodeling. Constitutive expression of NO can regulate osteoblastic metabolism, but over-produced NO is harmful to the bone cell. This study is aimed to investigate the protective role of low concentrations of NO on the high concentration of NO-induced osteoblastic insults and its possible mechanisms using human osteoblast-like MG63 cells as our experimental model. Sodium nitroprusside (SNP), a NO donor, was able to augmented the amounts of NO in MG 63 cells in a concentration-dependent manner. Administration of MG63 cells with 2 mM SNP for 16 hours resulted in the 6-fold increases of NO in MG63 cells and caused cell damages, including morphological alternations, decreases in cell viability and alkaline phosphatase activity as well as cell apoptosis. In contrast, pretreatment with 50 and 100 M SNP for 24 hours only increased 40% and 70% NO in MG63 cells and didn't lead to cell insult. Pretreatment of MG63 cells with 50 and 100 M SNP for 16 hours, and then with 2 mM SNP for another 16 hours significantly recovered the 2 mM SNP-caused decreases of cell viability and alkaline phosphatase activity. According to the morphological observation, pretreatment with 100 ck 2 mM SNP-induced morphological alternations. Analysis of apoptotic cells revealed that pretreatment with 100 M SNP significantly protected MG63 cells from 2 mM SNP-induced cell apoptosis. An immunoblotting result showed that pretreatment of MG63 cells with 100 M SNP decreased the basal and 2 mM SNP-induced Bax protein. The present study has shown that pretreatment with low concentrations of NO could promote cell tolerance against the high concentration of NO-induced osteoblast apoptosis through the suppression of pro-apoptotic Bax protein.

Keywords: Osteoblasts, Nitric oxide, Apoptosis

二、Introduction

Nitric oxide (NO) is a diatomic free radical and contributes to varieties of biological activities, including neurotransmission, vessel relaxation, immune responses (Moncada and Higgs, 1993). There are two constitutive forms of NO synthase (NOS) and one inducible form involving in the production of the diatomic free radical (Evans et al., 1995). NO is detectable in control osteoblasts and is also inducible after treatment of proinflammatory cytokines, mechanical stress, or fluid flow (Damoulis and Hauschka, 1994; Ralston et al., 1994; Riancho et al., 1995; Pitsillides et al., 1996; Johnson et al., 1996; Heflich et al., 1997; Zaman et al., 1999). NO has the potential role of modulating proliferation and differentiation of osteoblasts and bone resorption activity of osteoclasts, and of mediating the effects of proinflammatory cytokines and mechanical stress. The free radical is able to regulate bone maintenance and remodeling (Löwik et al., 1994; Pitsillides et al., 1995; Johnson et al., 1996; Chae et al., 1997; Hikiji et al., 1997; Chow et al., 1998).

NO has biphasic biological activities: low concentrations of NO play an important role in the maintenance of cell functions, but high concentrations of NO will cause cell apoptosis. Previous studies showed that NO protected oligodendrocytes, cardiomyocytes and hepatocytes from oxidative stress injury (Rosenberg et al., 1999; Stefanelli et al, 1999; Li et al., 1999). Osteoblasts play a crucial role in bone metabolism. NO is an important mediator for regulating osteoblastic metabolism (Collin-Osdoby et al., 1995). The present study is designed to investigate the protective effects of NO on oxidative stress-induced osteoblast apoptosis and their possible mechanisms from the aspects of cell morphology, cell viability, alkaline phosphatase (ALP) activity, immunoblotting analysis of Bax protein.

三、Materials and methods

Cell culture and treatment

Human osteoblast-like MG63 osteosarcoma cells were purchased from American Type Cells Collection (Rockville, MD, USA). MG63 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin and 100 streptomycin at 37°C in a humidified atmosphere of

[*] **此格式可至國科會網站（http://www.nsc.gov.tw）下載。**

5% CO_2. SNP (Sigma, St. Louis, MO, USA) was dissolved in a PBS buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) to 200 mM of stock solution and then stored at -20^oC for use in related experiments. MG63 cells were treated with various concentrations of SNP for different time intervals.

Determination of NO

The amounts of NO in MG63 cells were determined following the provided protocol in the Bioxytech NO assay kit (OXIS International, Inc, Portland, OR, USA).

Assays of cell viability and alkaline phosphatase activity

After treatment with SNP, viabilities of MG63 cells were determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described previously (Carmichael et al., 1987). Alkaline phosphatase (ALP) activities of MG63 cells were assayed by detecting the formation of *p*-nitrophenol, a product of *p*-nitrophenyl phosphate catalyzed by ALP, according to the procedure provided by Sigma Diagnostics Alkaline, Acid and Prostatic Acid Phosphotase kit (Sigma Diagnostics, Inc, St. Louis, MO, USA).

Analysis of apoptotic cells

By using a flow cytometer, apoptotic cells in MG63 cells were determined by detecting DNA fragments in nuclei stained by propidium iodide following the method of Nicoletti et al. (1991). After SNP treatment, MG63 cells were harvested and fixed in cold 80% ethanol. After a process of centrifugation and washing, the fixed cells were stained with propidium iodide and analyzed using a FACScan flow-cytometer (FACS Calibur, Becton Dickinson, San Joes, CA, USA) on the basis of a 560 nm dichroic mirror and a 600 nm band pass filter.

Immunoblotting analyses of Bax protein

MG63 cells were washed with PBS and lysed in ice cold RIPA buffer (Tris-HCl pH 7.2, 25 mM; SDS 0.1%; Triton X-100 1%; sodium deoxycholate 1%; NaCl 0.15 M; EDTA 1 mM) containing 1 mM of phenyl methyl sulfonyl fluoride (PMSF), 10 μg/ml of aprotinin, 1 mM of sodium orthovanadate and 5 μg/ml of leupeptin. Protein concentrations were determined with the BCA method (Pierce, Rockford, IL, USA). Cytosolic protein (100 μg) was subjected to sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane as described elsewhere (Chen and Ueng, 2000). Immunodetection of Bax protein was carried out using a rabbit polyclonal antibody against human Bax protein. Intensities of the immunoreactive bands were determined using an IS-100 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA) as described previously (Chen et al., 1998).

Statistical analysis

The statistical significance of the difference between control and drug-treated groups was evaluated by Duncan multiple rang test. A P value < 0.05 was considered statistically significant.

四、Results

SNP spontaneously or enzyme-metabolically releases NO under the presence of biological systems, reducing agents or visible light (Bates et al., 1991; Otsuka et al., 1998). Administration of MG63 cells with 10 M SNP for 24 hours
amounts of NO (Fig. 1). When the concentration of SNP reached to 50, 100 and 2000
NO in MG63 cells were significantly augmented by 40%, 70% and 6-fold.

Administration of MG63 cells with 10, 50 and 100 M SNP for 24 hours didn't influent cell viability and ALP activity (Table 1). However, tratemnt with 2 mM SNP for 16 hours caused 44% and 38% decreases in cell viability and ALP activity, respectively. Pretreatment of MG63 cells with 50 M SNP for 24 hours and then with 2 mM SNP for another 16 hours partialy blocked 2 mM SNP-caused cell death and inhibition of ALP activity, respectively. Pretreatment of 100
mM SNP-resulted in the decreases of cell viability and ALP activity.

Although treatment with 100
hours didn't affect morphologies of MG63 cells, the donor at 2 mM for 16 hour-treatment significantly alternate cell morphologies into shrinkage outlines (Fig. 2 B and 2C). Pretreatment of MG63 cells with 100 M SNP for 24 hours apparently decreased 2 mM SNP-induced cell damage (Fig. 2D).

SNP at 2 mM increased 55% apoptotic cells in MG63 cells, but at 100
3). Pretreament of MG63 cells with 100
for 24 hours and then with 2 mM SNP for another 16 hours significantly decreased almost 40% apoptotic cells induced by the high concentration of SNP.

Immunoblotting analysis using a rabbit polyclonal antibody against human Bax protein revealed that Bax protein was detectable in untreated MG63 cells (Fig. 4). Pretreatment of 100
significantly resulted in about 70% decrease of Bax protein in MG63 cells. Administration of MG63 cells with 2 mM SNP for 16 hours caused 3-fold increases of Bax protein. Pretreatment of 100
SNP for 24 hours inhibited the 2 mM SNP-induced levels of Bax protein in MG63 cells.

五、Discussion

Administration of SNP increases NO in human osteoblast-like MG63 cells and further causes oxidative stress to these cells. SNP is a clinical medicine used as a vasodilator for vasocardiac diseases because the NO donor can be decomposed to NO, a messaging molecule for relaxation of blood vessels (Bates et al., 1991). The detailed mechanism

of SNP decomposition to NO is still unknown. Several lines of evidence have reported that the NO donor can be decomposed to NO under the presence of biological systems, reducing agents or visible light (Bates et al., 1991; Kowaluk et al., 1992). The present study reveals that NO in MG63 cells was concentration-dependently augmented following SNP treatments. NO is a reactive oxygen species (Wink and Mitchell, 1998), whose increase of the free radical will result in the increase of oxidative stress in MG63 cells and the further modulation of osteoblastic physiology or pathophysiology.

NO could regulate osteoblast survival and metabolism. In parallel to the increase of NO in MG63 cells, SNP causes cell death. Osteoblasts play a crucial role in bone formation. Suppression of osteoblast viability or proliferation would interfere with the dynamic balance of bone remodeling, and lead to pathophysiological conditions of bone tissues (Collin-Osdoby et al., 1995). Previous studies reveal that NO, over-induced by proinflammatory cytokines, has a pathogenic role when it acts as a mediator of these cytokines for the stimulation of osteoblast death (Damoulis et al., 1997; Armour et al., 1999; Mogi et al., 1999). Our previous study had also shown that NO from an extracellular NO donor has cytotoxic effects on rat osteoblasts as the intracellular NO induction (Chen et al., 2001). ALP is a marker enzyme for osteoblast metabolism (Collin-Osdoby et al., 1995). Treatment of MG63 cells with SNP results in concentration- and time-dependent decreases of ALP activity. The reduction of ALP activity caused by NO may be partially due to the cytotoxic effects of NO on MG63 cells. However, NO at high concentration levels would modulate osteoblast metabolism and lead to cell death.

NO has a biphasic role in modulating cell function. Low concentration of NO is benefit to maintain normal cellular physiology, but high concentration of NO will damage cells (Collin-Osdoby et al., 1995). The amounts of NO released by SNP are dependent on the dosage of the NO donor. SNP at 2 mM releases massive amounts of NO, increases oxidative stress, and leads to cell apoptosis of human osteoblast-like MG63 cells. SNP at 100
much less amounts of NO than at 2 mM. The level of NO released by 100
human MG63 cells but promotes the tolerance of the osteoblast-like cells against oxidative stress. Therefore, pretreatment with low concentration of SNP can protect human MG63 cells from more oxidative stress-induced cell apoptosis. Bax protein is a proapoptotic protein. Our previous study has shown that high concentration of NO induces osteoblast apoptosis through induction of Bax protein (Chen et al., 2001). The present study reveals that SNP at low concentration inhibits the basal level of Bax protein and completely suppresses high concentration of SNP-caused increase of the protein. From the present data, we propose that NO could protect osteoblast-like cells from oxidative stress-induced cell apoptosis through suppression of Bax protein. During the inflammation, appropriate elevation of intracellular NO may protect osteoblasts against more oxidative stress-induced cell death.

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