附件：封面格式

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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※ 活化氧物質在炎性骨流失中所伴演的角色 ※

※ 及其機制的探討 ※

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計畫類別：☑個別型計畫　□整合型計畫

計畫編號：NSC－89－2320－B－002－111

執行期間：88 年 8 月 1 日至 89 年 7 月 31 日

計畫主持人：王至弘

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計畫參與人員：

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執行單位：台大醫學院骨科

中　華　民　國　90 年 3 月 14 日

行政院國家科學委員會專題研究計畫成果報告

活化氧物質在炎性骨流失中所伴演的角色及其機制的探討
Role of reactive oxygen species in inflammatory osteolysis and its mechanism

計畫編號：NSC 89-2320-B-002-111
執行期限：88 年 8 月 1 日至 89 年 7 月 31 日
主持人：王至弘　　國立台灣大學醫學院附設醫院骨科部

一、中文摘要

活化氧物質和一氧化氮產生於炎性病程中，並伴演重要的角色。一氧化氮引起的骨母細胞毒性與活化氧物質有關，兩者之間的平衡，影響骨細胞的代謝，本計畫使用一氧化氮供給藥物和炎性細胞因子，佐以活化氧物質，處理骨母細胞，用以研究其聯合作用對骨母細胞代謝，細胞毒性和一氧化氮生成的作用。研究顯示活化氧物質和炎性細胞因子會促進一氧化氮對骨母細胞的作用，加速細胞凋亡。這個過程和一氧化氮的過度產生，促進骨母的細胞化有關；因此控制一氧化氮和活化物質的產生，可以治療炎性骨細胞凋亡的現象。

關鍵詞：活化氧物質、一氧化氮、炎性細胞因子、骨母細胞、細胞毒性、細胞凋亡

Abstract

Reactive oxygen species (ROS) and nitric oxide (NO) are generated in a variety of conditions and they play an important role in the inflammatory response. Reactive oxygen species are involved in NO-induced cellular toxicity in osteoblasts. A balance between NO and reactive oxygen species seems to be associated with the effects of NO on bone metabolism. Thus the interaction between oxygen reactive species and nitric oxide may have important potential implication in their actions on osteoblasts.

We propose to use NO donor to supply exogenous NO and cytokine-induced endogenous NO in addition to hydrogen peroxide to study the cellular toxicity of NO in osteoblasts. Reactive oxygen species and proinflammatory cytokines (interleukin-1□ and TNF-□) are involved and potentiate the effects of NO on osteoblast cell death and metabolism. Knowledge gained from this study can be extended to the understanding the roles of reactive oxygen species and nitric oxide in bone and joint diseases and treatment of such diseases based on pharmacological manipulation of the NO pathway and antioxidants.

Keywords: reactive oxygen species, nitirc oxide, cytokines, osteoblast, cellular toxicity

Introduction

Stimulation of NO production in bone by proinflammatory cytokines and release of NO from the blood vessels, neutrophils and activated macrophages in the inflammatory processes raise the possibility that *NO* may be involved as a mediator of bone disease in conditions, such as rheumatoid arthritis, inflammation-mediated osteopenia, tumor associated osteolysis, postmenopausal osteoporosis, sepsis, and aseptic loosening of joint prostheses[1,3,6].

Reactive oxygen species (ROS) are generated in a variety of conditions and they play an important role in the inflammatory response[4]. NO may react with oxygen-derived free radicals such as superoxide anions to form a cascade of highly reactive species, including the peroxynitrite anion and the hydroxyl radical. Production of such toxic moieties may contribute to the tissue damage[4,9]. Reactive oxygen species are involved in NO-induced cellular toxicity in osteoblasts and a balance between NO and reactive oxygen species seems to be associated with the effects of NO on bone metabolism. Thus the interaction between oxygen reactive species and nitric oxide may have important potential implication in their actions on osteoblasts.

At present, it is not yet known about the toxicity mechanisms stimulated by nitric oxide on osteoblasts[2,5]. We propose to use NO donor to supply exogenous NO and cytokine-induced endogenous NO in addition to hydrogen peroxide to study the cellular toxicity of NO in osteoblasts.

Materials and methods

Cell treatment

(1) Interaction between reactive oxygen species and exogenous nitric oxide

Human MG-63 osteoblasts cultured with DMEM were treated with Sodium Nitroprusside with or without H_2O_2. Sodium nitroprusside (SNP) was used as NO donor. The effects of SNP with or without H_2O_2 on the cell viability and ALP activity were determined.

(2) Interaction between reactive oxygen species and endogenous nitric oxide

Pro-inflammatory cytokines were increased in inflammation, and they could stimulate the endogenous NO production of osteoblasts[1]. MG-63 osteoblasts were stimulated with recombinant human interleukin-1 β and TNF-α to release endogenous nitric oxide. Then SNP and/or H_2O_2 were added to the cell cultures to study the combined effects of inflammatory cytokines, and reactive oxygen species on osteoblasts.

MTT assay MG-63 osteoblast cell viabilities after 4 hours of treatments was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye to test the mitochondria[7]

Alkaline phosphotase activity MG-63 osteoblasts were plated onto a 96-well plate. After various treatments, cell lysate was incubated with 100mM p-nitro-phenyl phosphate as a substrate at 37 degrees for 10 min. The optical density of p-nitrophenol, a reaction product, was determined at 405 nm using ELISA reader.

Determination of nitrite concentration 100□L of culture medium was reacted with equal amount of Griess reagent for 10 min in room temperature. Absorbance was measured at 540 nm and then nitrite concentration was quantified by using a standard curve of sodium nitrite.

Fluorescence microscopy for morphological investigation For fluorescence microscopy, cells were stimulated according to experimental protocols, collected and followed by fixation in methanol/acetone (1/3,v/v) solution for 5min and washed with PBS. Then fixed cell were stained with 0.1mg/ml Hoechst 33258 for 10min at dark . Cells were observed and photographed under a Nikon fluorescence microscope

Evaluation of cellular apoptosis by flow cytometric analysis Monolayer cultures were treated and harvested by trypsinization after various exposure times and washed with ice-cold phosphate-buffered saline. Monodispersed cells were fixed with 70% ethanol at – 20 °C for at least 1 hr. DNA content was measured after simultaneous cell treatment by RNase A (Sigma) (1 mg/ml, in PBS) and staining with 0.5 ml of propidium iodide. Fluorescence was quantitated after laser excitation of the fluorescent dye by a FACScan flow cytometry[8]. The number of apoptotic cells was presented as a percentage of hypodiploid nuclei

Results

NO released from SNP is cytotoxic for MG-63 osteoblasts

Freshly prepared SNP solution in DMEM at concentrations more than 0.1mM caused a marked loss of viable MG-63 osteoblasts in a dose-dependent manner (Fig. 1). Addition of hydrogen peroxide increased the cytotoxicity of SNP (Fig 2).

Effect of exogenous NO on the activity of ALP

Treatment of MG-63 osteoblasts with low dose (subtoxic dose) of SNP resulted in a slight increase in ALP activity (Fig 3), which suggested low dose NO might promote osteoblast differentiation. Addition of hydrogen peroxide might increase the activity of alkaline phosphotase in 4 hrs (Fig 4), but the effect was inhibitory after 24 hrs.

Production of NO by MG-63 osteoblasts

Single treatment with each cytokine (interleukin-1 β or TNF-α) did not stimulate MG-63 osteoblasts to produce NO. However, a significant amount of NO was produced when stimulated with the combination of SNP and inflammatory cytokine (Fig. 5). Hydrogen peroxide in combination with SNP increased the production of NO as compared to hydrogen peroxide or SNP treatment alone (Fig. 6). These results indicate that inflammatory cytokines and ROS potentiate the effect of SNP to elevate NO level.

SNP-induced cytotoxicity involves an apoptotic process Cells treated with 0.5 mM and 1mM SNP resulted in apoptotic cell death, as shown by Hoechst 33258 dye staining using fluorescence microscopy. Hydrogen peroxide (1mM) and inflammatory cytokines (interleukin-1 β and TNF-α) promoted the SNP-induced MG-63 osteoblast apoptosis (Fig. 7), as measured by PI flow cytometry.

Evaluation of the project outcomes

(1) Reactive oxygen species are involved in NO-induced cellular toxicity in osteoblasts. A balance between NO and reactive oxygen species seems to be associated with the effects of NO on bone metabolism.

(2) In osteoblats, pro-inflammatory cytokines induce NO and hydrogen peroxide productions which may account for the cellular cytotoxity of pro-inflammatory cytokines due to the interaction between NO and reactive oxygen species. Thus pro-inflammatory cytokines, nitric oxide, and reactive oxygen species are involved in the pathogenesis of bone and joint diseases, including inflammation, arthritis, osteoporosis, sepsis, ligament healing, and aseptic loosening of joint prosthesis.

(3) Exogenous and endogenous NO production and reactive oxygen species were involved in the mechanisms of osteoblast cell death and differentiation. The osteoblast cell death in this model invlolved a apoptotic process.

(4)NO and reactive oxygen species were implicated in inflammatory osteolysis. Further studies are needed to elucidate the apoptotic pathways.

References:

(1) Damoulis PD, and Hauschka PV: Biochem Biophys Res Commun 201: 924, 1994.

Fig. 6. NO production of osteoblasts treated by SNP in combination with H2O2

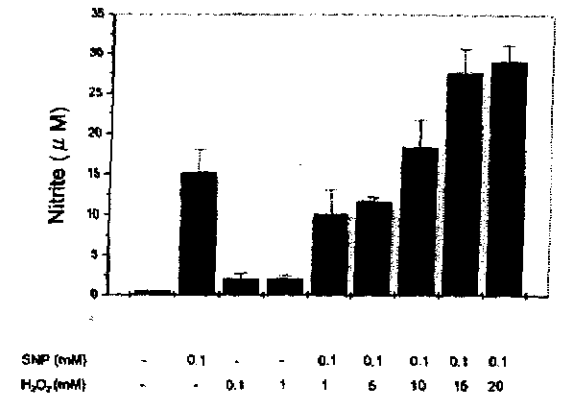
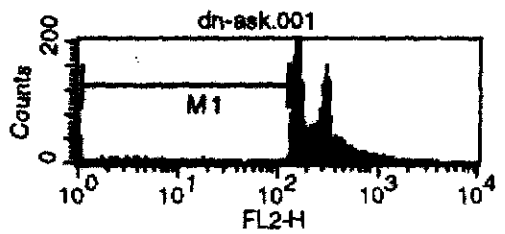


Fig. 7 PI Flowcytometry analysis of cellular apoptosis.

(A) Control



(B) Cells treated with SNP(0.1mM) and H2O2 (1mM)