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行政院國家科學委員會補助專題研究計畫成果報告

一氧化氮誘導骨母細胞凋零研究 -探討 Caspases 所扮演之角色(1/2)

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執行期間：91 年 08 月 01 日至 92 年 07 月 31 日

計畫主持人：陳瑞明

共同主持人：

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行政院國家科學委員會專題研究計畫成果報告

一氧化氮誘導骨母細胞凋零研究-探討 Caspases 所扮演之角色 (1/2)

STUDY ON NITRIC OXIDE-INDUCED OSTEOBLAST APOPTOSIS: THE ROLE OF CASPASES

計畫編號：NSC 91-2314-B-038-031-
執行期限：91 年 08 月 01 日至 92 年 07 月 31 日
主持人：陳瑞明　台北醫學大學醫學系
計畫參與人員：林怡伶、黃景怡　台北醫學大學醫學系

Abstract

Nitric oxide contributes to osteoblast metabolism. This project is designed to determine the role of different types of caspases in the nitric oxide-induced osteoblast apoptosis using primary osteoblasts from neonatal rat calvariae as the experimental model. The first year of this project determined the role of the mitochondria-dependent caspase activation pathway, including apoptotic factors and caspase-3, in the nitric oxide-induced osteoblast apoptosis. Exposure of osteoblasts to sodium nitroprusside, a nitric oxide donor, significantly increased lactate dehydrogenase release and decreased cell viability in concentration- and time-dependent manners. Sodium nitroprusside concentration- and time-dependently caused DNA fragmentation in osteoblasts. In parallel to sodium nitroprusside-induced osteoblast apoptosis, this nitric oxide donor increased the amounts of intracellular reactive oxygen species. However, ascorbic acid and N-acetyl cystein could not block sodium nitroprusside-induced reactive oxygen species in rat osteoblasts. Administration of sodium nitruprusside significantly reduced the membrane potential and NADH oxidase activity in osteoblast mitochondria. Immunoblotting analysis revealed that sodium nitruprusside decreased the levels of Bcl-2 protein in osteoblasts. The activities of caspase-3 were time-dependently increased following sodium nitroprusside treatment. Administration of sodium nitroprusside increased the levels of 17 kD activated subunits of caspase-3. The present study has shown that nitric oxide released from sodium nitroprusside could induce osteoblast insults and apoptosis, and this might be involved by modulating intracellular oxidative stress, mitochondrial functions, anti-apoptotic Bcl-2 protein and caspase-3.

Keywords: Osteoblasts, Nitric oxide, Apoptosis, Caspase-3

Introduction

Nitric oxide (NO), one of reactive oxygen species (ROS), contributes to a variety of biological activities, including vaso-regulation, neuronal transmission, immune response, or cell apoptosis (Moncada et al., 1991; Wink et al., 1998; Brüne et al., 1999; Chang et al., 2002). For modulating bone formation and resorption, NO plays an important role in bone remodeling (Collin-Osdoby et al., 1995; Chae et al., 1997). Osteoblasts, which mediate bone formation in bone metabolism, can constitutively produce NO (Riancho et al., 1995; Collin-Osdoby et al., 1995). In response to the stimulation of pro-inflammatory cytokines, large bodies of NO will be synthesized in osteoblasts (Riancho et al., 1995; Mancini et al., 2000). NO has the biphasic effects on osteoblasts. As an effective mediator, the constitutive

expression of NO is involved in the proliferation and differentiation of human and rodent osteoblasts (O'Shaughnessy et al., 2000). As a death effector, the over-produced NO can cause osteoblast injury or even death (Armour et al., 1999; Chen et al., 2002).

Apoptosis, also named programmed cell death, is not only an evolutionarily conserved form of physiologic cell death in normal development and homeostasis but also contributes to the pathophysiology of tissue/cell injury (Goyal, 2001; Rathmell and Thompson, 2002). When associated with proliferation and differentiation, apoptosis determines the size of osteoblast population and is involved in bone remodeling in the postanal and adult skeleton (Hock et al., 2001). NO is a critical bioregulator to induce cell apoptosis (Chung et al., 2001; Chang et al., 2002). The over production of NO in osteoblasts responsive to pro-inflammatory cytokine stimulation would lead to osteoblast death (Damoulis and Hauschka, 1999; Mogi et al., 1999). Administration of NO donors such as sodium nitroprusside (SNP) would release massive levels of NO and induce osteoblast apoptosis (Messmer et al., 1996; Chen et al., 2002).

There are multiple mechanisms involved in cell apoptosis. An increase in the levels of reactive oxygen species can enhance oxidative stress and then induce cell insults (Shackelford et al., 2000). Administration of NO donors and peroxynitrite, an oxidative metabolite of NO and superoxide, would enhance cellular oxidative stress and promote cell apoptosis (Wei et al., 2000; Chung et al., 2001; Del Carlo and Loeser, 2002). Mitochondria are critical for the maintenance of cell functions. The disruption of mitochondrial activity would lead to cell dysfunction or even death (Yu et al., 2002). NO has been reported to interfere with mitochondrial functions through modulating the membrane potential and to promote the release of apoptotic factors, including, including reactive oxygen species and cytochrome c, from this organelle to the cytoplasm (Hortelano et al., 1999; Umansky et al., 2000; Pearce et al., 2001). Bcl-2 is an anti-apoptotic protein, the decrease in cellular levels would drive cells undergoing apoptosis (Srivastava et al., 1999; Brüne et al., 1999; Cheng et al., 2001). Caspases are constitutively present in cells as nonactive zymogens and require proteolytic cleavage into the catalytic active heterodimeter. Initiator or signaling caspases are activated by an upstream signal transducing step thus processing downstream or effector/executor caspases that in turn are implicated in the degradation of multiple substrates (Cohen, 1997). However, the roles of cellular oxidative stress, mitochondrial functions, Bcl-2 and caspase-3 in NO-induced osteoblast apoptosis are still unknown. This study is aimed to investigate if the NO-induced osteoblast apoptosis is involved by intracellular reactive oxygen species, mitochondrial activities, Bcl-2 protein and caspase-3 using neonatal rat calvarial osteoblasts as the experimental model.

Materials and Methods

Preparation of rat osteoblasts

Primary osteoblasts were prepared from 3-day-old Wistar rat calvaria according to the method of Partridge et al. (1981). Osteoblasts were seeded in Dulbecco's modified Eagle medium (Gibco, BRL, Grand Island, NY, USA) supplemented with 10 % heat-inactivated fetal bovine 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. Osteoblasts were grown to a confluence prior to the drug treatment. Only the first passage of rat osteoblasts was used in the present study.

Drug treatment

Sodium nitroprusside (SNP) purchased from Sigma Corporation (St. Louis, MO, USA) was freshly dissolved in phosphate-based saline (PBS) buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and protected from light. The concentration- and time-dependent effects of SNP on osteoblasts were determined first.

Quantification of lactate dehydrogenase

The amounts of lactate dehydrogenase released in the culture medium by osteoblasts were determined to evaluate the cytotoxicity of SNP to these cells. Osteoblasts (1×10^5) were seeded in 24-well tissue culture plates (Corning Costar Corporation, Cambridge, MA, USA). After administration of SNP, the culture medium was collected and centrifuged. The amounts of lactate dehydrogenase in supernatants were analyzed using a model 7450 automatic autoanalyzer system of Hitachi Ltd., Tokyo, Japan.

Assay of the membrane integrity

The integrity of osteoblast membrane was determined by the trypan blue exclusion method to evaluate the cytotoxicity of SNP. Briefly, osteoblasts (2×10^5) were cultured in 24-well tissue culture plates. After SNP administration, osteoblasts were trypsinized using 0.1 % trypsine-EDTA (Gibco, BRL, Grand Island, NY, USA) and harvested in 1 x PBS buffer. Following centrifuging and washing, osteoblasts were suspended in 1 x PBS buffer and stained with equal volume of trypan blue dye. The fractions of dead cells with blue color in osteoblasts were counted in a reverse-phase microscope.

Analysis of cellular DNA fragmentation

DNA fragmentation in osteoblasts were quantified by detecting BrdU-labeled histone-associated DNA fragments in the cytoplasm of cell lysates according to the instruction of the cellular DNA fragmentation ELISA kit (Boehringer Mannheim, Indianapolis, IN, USA). Briefly, osteoblasts (2×10^5) were sub-cultured in 24-well tissue culture plates and labeled with BrdU for over night. The cells were harvested and suspended in the culture medium. One hundred micrometer of cell suspension was added in each well of 96-well tissue culture plates. The cells were co-cultured with SNP for another 8 hours at 37 °C in a humidified atmosphere of 5 % CO_2. The amounts of BrdU-labeled DNA in the cytoplasm were quantified by an Anthos 2010 microplate photometer (Anthos Labtec Instruments GmbH, Lagerhausstrasse, Wals/Salzburg, Aus) at a wavelength of 450 nm.

Determination of intracellular reactive oxygen species

The levels of intracellular reactive oxygen species were quantified following the method as described previously (Liu et al., 2001). Briefly, 5×10^5 osteoblasts were cultured in 12-well tissue culture plates for overnight, and then co-treated with SNP and 2',7'-dichlorofluorescin diacetate, an reactive oxygen species sensitive dye. After drug treatment, osteoblasts were harvested and suspended in 1 x PBS buffer. The relative fluorescence intensity in osteoblasts was quantified by a flow cytometer (FACS Calibur, Becton Dickinson, San Joes, CA, USA).

Quantification of mitochondrial membrane potential

The membrane potential of mitochondria in osteoblasts was determined according to the method of Chen (1988). Briefly, osteoblasts (5×10^5) were seeded in 12-well tissue culture plates for overnight and then treated with SNP for different time intervals. 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, the cell pellets were suspended with 1 x PBS buffer. The fluorescent intensities in osteoblasts were analyzed by a flow cytometer (FACS Calibur, Becton Dickinson, San Joes, CA, USA).

Assay of NADH-dependent dehydrogenase activity

The activity of NADH-dependent dehydrogenase were determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay following the method of Wu et al. (2003) to evaluate the effect of SNP on mitochondria. Briefly, ten thousand macrophages were seeded in 96-well tissue culture clusters for overnight. After drug treatment, cells were cultured with a new medium containing 0.5 mg/ml 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for another three hours. The blue formazan product in cells was dissolved in dimethyl sulfoxide and measured spectrophotometrically at a wavelength of 550 nm.

Caspase-3 fluorometric assay

Caspase-3 activity was determined by fluorometric reaction according to the standard protocol in the caspase-3 fluorometric assay kit (R&D Systems, Inc., Minneapolis, MN, USA). After SNP treatment, the cytosolic extracts of osteoblasts were prepared by lysing the cells in a buffer containing 1 % Nonidet P-40, 200 mM NaCl,20 mMTris/HCl, pH 7.4, 10 μg/ml leupeptin, 0.27 U/ml aprotinin and 100 μM PMSF. The cell lysate (100 μg total protein) was incubated at 37 °C for 3 hours with 50 μM the fluorogenic substrate, DEVD-AFC, in 200 μl cell-free system buffer (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 1mM dithiothreitol). The release of fluorescent 7-amino-4-trifluoromethyl coumarin was measured by LS55 Luminescence Spectrometer (Perkin Elmer Instrutments, Shelton, CT, USA).

Gel electrophoresis and immunoblotting analysis

After SNP treatment, osteoblasts were washed with 1 x PBS buffer, and the 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, 1 mM EDTA). To avoid protein degradation, the RIPA buffer contained a mixture of proteinase inhibitors, including 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate and 5 μg/ml leupeptin. Protein concentrations were quantified by a bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Cytosolic proteins (100 μg per well) were subjected to Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to nitrocellulose membrane as described elsewhere (Chen et al., 2000). The membranes were blocked with 5 % non-fat milk at 37 °C for 1 hour. Immunodetection of Bcl-2 and caspase-3 was carried out using mouse monoclonal antibody against rat Bcl-2 and caspase-3 (Transduction Laboratories, Lexington, KY, USA). Cellular β-actin protein was immunodetected by a mouse monoclonal antibody against mouse β–actin (Sigma, Saint Louis, MI, USA) as an internal standard. Intensities of the immunoreactive bands were determined using an UVIDOCMW Version 99.03 digital imaging system (Uvtec Limited, Cambridge, England, UK).

Statistical analysis

The statistical significance of the difference between control and drug-treated groups was evaluated by the Student's *t*-test. A *P*-value less than 0.05 was considered as statistically significant. The statistical difference between groups was considered significant when the *P* value of the Duncan's multiple rang test was less than 0.05.

Results

Cytotoxicity of SNP to osteoblasts was assayed by analyzing the release of lactate dehydrogenase and the membrane integrity (Tables 1 and 2). Exposures of osteoblasts to 1.5 and 2 mM SNP for 16 hours resulted in 85 % and 2.5-fold increases of lactate dehydrogenase in the culture medium, respectively (Table 1). SNP at 0.5 and 1 mM did not affect the release of lactate dehydrogenase. Analysis of trypan blue exclusion revealed that administration of osteoblasts with 1.5 and 2 mM SNP for 16 hours decreased 38 % and 62 % cell viability, respectively (Table 1). Exposure of osteoblasts to 0.5 and 1 mM SNP did not influence cell viability.

Administration of 2 mM SNP for 8 and 16 hours significantly increased 65 % and 2.5-fold the release of lactate dehydrogenase from osteoblasts to the culture medium, respectively (Table 2). The level of lactate

dehydrogenase in culture medium was not affected by SNP treatment for 4 hours. Exposure of osteoblasts to 2 mM SNP for 8 and 16 hours significantly reduced 40 % and 55 % cell viability, respectively (Table 2). After administration of SNP for 4 hours, the cell viability was not changed.

DNA damage was quantified to determine if osteoblastss underwent apoptosis following SNP treatment. Exposure of osteoblasts to 1.5 and 2 mM SNP for 16 hours significantly increased 92 % and 3.5-fold DNA fragmentation (Fig. 1). SNP at 0.5 and 1 mM did not cause DNA damage. When exposed to 2 mM SNP for 8 and 16 hours, the ratio of DNA fragmentation in osteoblasts was significantly augmented by 65 % and 3.5-fold. Exposure of osteoblasts to 2 mM SNP for 4 hours did not lead to DNA fragmentation.

After SNP treatment, the levels of intracellular reactive oxygen species were analyzed to determine if SNP induced intracellular reactive oxygen species in osteoblasts (Fig. 2). Exposure of osteoblasts to SNP for 1 hour significantly increased 76 % intracellular reactive oxygen species (Fig. 2). After administration of SNP for 2 and 4 hours, this NO donor resulted in 3.2- and 5.3-fold increases in the levels of intracellular reactive oxygen species.

Ascorbic acid and N-acetyl cystein, two typical antioxidants, were used to block SNP-induced intracellular reactive oxygen species (Table 3). Exposure of osteoblasts to SNP significantly increased about 5-fold intracellular reactive oxygen species. The basal levels of intracellular reactive oxygen species in osteoblasts exposed to ascorbic acid and N-acetyl cystein were decreased by 54 % and 63 %, respectively. However, combined treatment of SNP with ascorbic acid or N-acetyl cystein could not abolish SNP-induced intracellular reactive oxygen species (Table 3).

The membrane potential and activity of NADH-dependent dehydrogenase were determined to evaluate the effect of SNP on mitochondrial function (Fig 3). Exposure of osteoblasts to SNP for 1 hour did not affect the membrane potential of mitochondria (Fig. 3). When the administered time intervals achieved 2 and 4 hours, SNP significantly resulted in 16 % and 36 % decreases in the membrane potential of osteoblast mitochondria. The activities of NADH-dependent dehydrogenase in osteoblast mitochondria exposed to 2 mM SNP for 8 and 16 hours were decreased by 18 % and 58 %, respectively. However, administration of osteoblasts with 2 mM SNP for 4 hours did not affect NADH-dependent dehydrogenase activity.

Bcl-2 protein in osteoblasts was immunodetected and quantified to determine the effect of SNP on theis anti-apoptotic protein (Fig 4). In untreated osteoblasts, Bcl-2 protein was detectable (Fig. 4A, *top panel*, lane 1). After SNP administration, the levels of Bcl-2 protein were significantly decreased (lane 2). The expression of β-actin was used as the internal standard (Fig. 4A, *bottom panel).* Intensities of the immunoreactive protein bands were analyzed using a digital imaging system (Fig. 4B). SNP significantly reduced 82 % Bcl-2 protein levels in osteoblasts.

Following the increase in intracellular calcium concentration, administration of osteoblasts with SNP for 1, 3, 6 and 12 hours caused significant increases in caspase-3 activity and protein, respectively (Figs. 5 and 6).

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Table 1 Concentration-dependent effects on the cytotoxicity of sodium nitroprusside to rat osteoblasts

SNP, mM	Lactate dehydrogenase (U/L)	Cell viability (cell number x 10^3)
0	41 ± 11	188 ± 44
0.5	38 ± 10	187 ± 38
1	35 ± 8	179 ± 45
1.5	76 ± 8*	116 ± 18*
2	104 ± 13*	72 ± 21*

Rat osteoblasts were exposed to 0.5, 1, 1.5 and 2 mM sodium nitroprusside (SNP) for 16 hours. The amounts of lactate dehydrogenase released from osteoblasts to culture medium were analyzed by an autoanalyzer as described in Materials and method. Cell viability was determined by the trypan blue exclusion method. Each value was expressed as Mean ± SEM for n = 12. *Values were considered to have statistical difference from the respective control, $P < 0.05$.

Table 2 Time-dependent effects on the cytotoxicity of sodium nitroprusside to rat osteoblasts

Time, h	Lactate dehydrogenase (U/L)	Cell viability (cell number x 10^3)
0	37 ± 10	179 ± 31
4	30 ± 6	167 ± 58
8	61 ± 9*	107 ± 30*
16	93 ± 11*	81 ± 19*

Rat osteoblasts were exposed to 2 mM sodium nitroprusside for 0, 4, 8 and 16 hours. The amounts of lactate dehydrogenase released from osteoblasts to culture medium were analyzed by an autoanalyzer as described in Materials and method. Cell viability was determined by the trypan blue exclusion method. Each value was expressed as Mean ± SEM for n = 12. *Values were considered to have statistical difference from the respective control, $P < 0.05$.

Table 3 Effects of ascorbic acid and N-acetyl cysteine on sodium nitroprusside-induced intracellular reactive oxygen species in rat osteoblasts

	iROS, folds of control
Control	1
SNP	4.68 ± 0.36*
AA	0.46 ± 0.02*
AA + SNP	5.83 ± 0.77*
NAC	0.37 ± 0.03*
NAC + SNP	6.13 ± 1.02*

Rat osteoblasts were exposed to 2 mM sodium nitroprusside (SNP), 30 μM ascorbic acid (AA), 1 mM N-acetyl cysteine (NAC), a combination of AA and SNP and a combination of NAC and SNP for 4 hours. The amounts of intracellular reactive oxygen species (iROS) were quantified by the flow cytometric method. Each value was expressed as Mean ± SEM for n = 6. *Values were considered to have statistical difference from the respective control, $P < 0.05$.

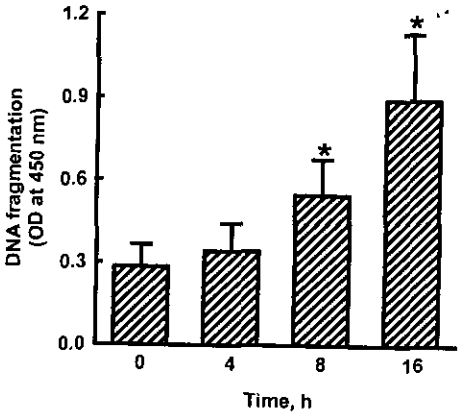

Fig. 1 Time-dependent effects of SNP on DNA fragmentation of rat osteoblasts.

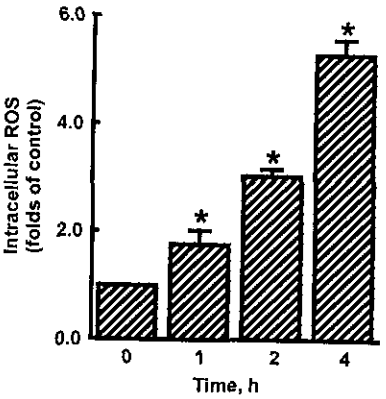

Fig. 2 Time-dependent effects of SNP on intracellular reactive oxygen species of rat osteoblasts.

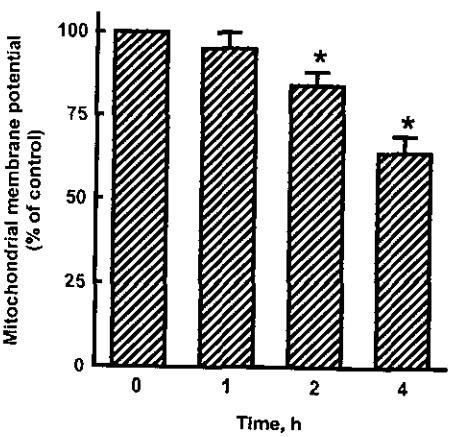

Fig. 3 Time-dependent effects of SNP on mitochondrial membrane potential of rat osteoblasts.

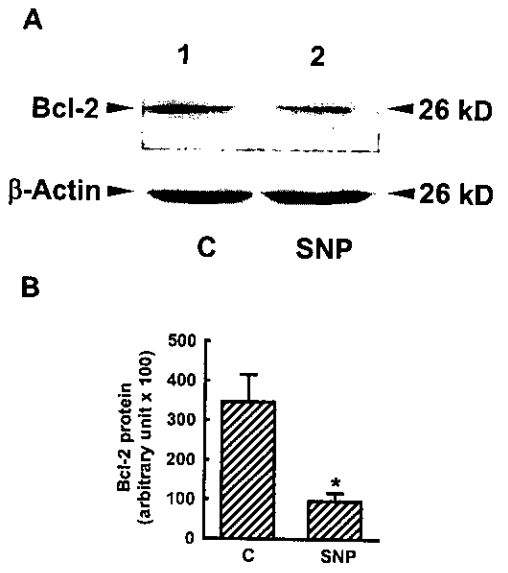

Fig. 4 Effects of SNP on Bcl-2 protein of rat osteoblasts.

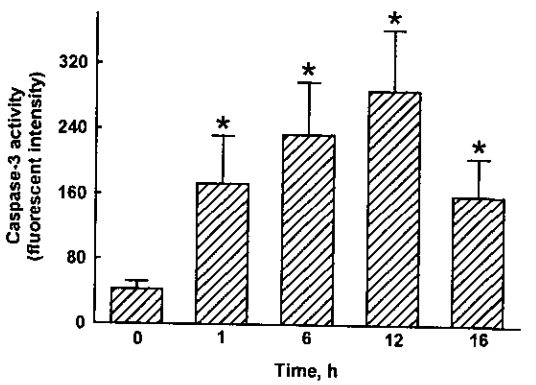

Fig. 5 Efffects of SNP on caspase-3 activity of rat osteoblasts.

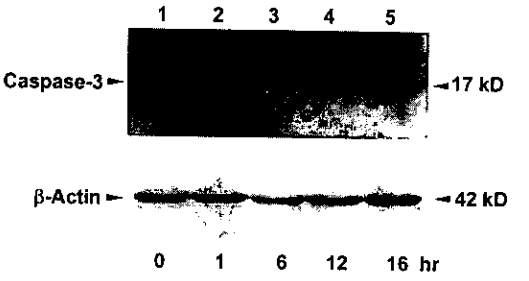

Fig. 6 Effects of SNP on the activated subunit of caspase-3 protein.