Full Paper

Nitric Oxide Induces Apoptosis in Mouse C2C12 Myoblast Cells

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Abstract. To investigate whether nitric oxide (NO) induces apoptosis in myoblast cells, the effect of the sodium nitroprusside (SNP), NO donor, on the apoptosis of mouse C2C12 myoblast cells was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, 4,6-diamidino-2-phenylindole (DAPI) staining, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, DNA fragmentation assay, reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis, and caspase-3 enzyme assay. Mouse C2C12 myoblast cells treated with SNP exhibited several apoptotic features. SNP increased p53 expression and bax expression. SNP also enhanced caspase-3 enzyme activity. The data show that NO may induce apoptotic cell death in myoblast cells through the activation of p53-, bax-, and caspase-dependent intracellular death-related pathways.

Keywords: nitric oxide, apoptosis, C2C12 myoblast cell

Introduction

Nitric oxide (NO) is a radical compound generated during the transformation of L-arginine to citrulline by the enzyme nitric oxide synthase (NOS) in living cells. NO is well known to play an important role in immune function, regulation of vascular tone, neuronal plasticity, and intracellular signaling. Recently, NO has been shown to function as an endogenous modulator of muscle physiology including vasodilation, metabolism, and contraction (1). However, the excessive production of NO in the skeletal muscles induces oxidative injury and apoptotic cell death, resulting in muscle wasting in muscular dystrophy, cachexia, extreme disuse, and aging (2, 3).

Apoptosis, also known as programmed cell death, is a form of cell death that serves to eliminate dying cells in the proliferating or differentiating cell populations (4). Apoptosis thus plays a crucial role in normal development and tissue homeostasis (5). Inappropriate or excessive apoptosis has been implicated in the pathogenesis of

several diseases (6, 7). The characteristic morphological changes of apoptosis are cell shrinkage, chromatin condensation, membrane blebbing, internucleosomal DNA fragmentation, and formation of apoptotic body (4). Several genes have been demonstrated to be implicated in the regulation of apoptosis. p53 is a very short-lived transcriptional activator that induces apoptosis and plays a key role in regulating apoptosis (8). It was reported that the activation of p53 regulates the expression of bax (9). Bax is a proapoptotic member of the Bcl-2 family of intracellular proteins, which can alter the permeability of mitochondrial membrane and trigger caspase cascade activation (10, 11). The caspases, a class of cysteine proteases, are considered as central players of the apoptotic process and trigger a cascade of proteolytic cleavage events in mammals. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many proteins (12).

C2C12 cells are myoblastic cells that exhibit most of the characteristics of normal myoblastic cells (13). C2C12 cells are commonly used as a model for studying muscle cell growth and differentiation. In the present

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study, it was investigated whether NO induces apoptosis of mouse C2C12 myoblast cells. Cells were treated with sodium nitroprusside (SNP), a chemical NO donor, and the following assays were performed: NO detection assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, 4,6-diamidino-2-phenylindole (DAPI) staining, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, DNA fragmentation assay, reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis, and caspase-3 enzyme assay.

Materials and Methods

Cell culture

The mouse C2C12 myoblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL) at 37°C in 5% CO₂, 95% O₂ in a humidified cell incubator, and the medium was changed every 2 days.

Measurement of NO generation

To examine the effect of SNP on NO synthesis, the amount of nitrite (NO₂⁻) in cell-free culture supernatant was measured using a commercial NO detection kit (iNtRON, Inc., Seoul, Korea). Briefly, after treatment with 50 or 100 μM SNP for 12 h, 100 μl supernatant was collected. N1 buffer (50 μl) was added to each well and the plate was incubated at room temperature for 10 min. N2 buffer was then added and the plate was incubated at room temperature for 10 min. The absorbance was measured at 450 nm. The nitrite concentration was calculated from a nitrite standard curve.

MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit as per the manufacturer's protocol (14). To determine the dose- and time-dependent cytotoxicity of NO, cells were treated with SNP at concentrations of 1, 5, 10, 50, 100, 500, and 1000 μM for 6, 12, and 24 h and MTT assays were performed. Control cells were not treated with SNP. A 10-μl aliquot of the MTT labeling reagent was added to each well and the plates were incubated for 4 h. Solubilization solution of 100 μl was added to each well and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at the test wavelength of 595 nm and the reference wavelength of 690 nm. Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and the test wavelength. The percent

viability was calculated as (O.D. of drug-treated sample / control O.D.) × 100.

Flow cytometric analysis

Flow cytometric analysis was performed as previously described (14). After treatment with 50 or 100 μM SNP for 12 h, cells were collected and fixed by incubating with 75% ethanol in PBS at -20°C for 1 h. Afterwards, the cells were incubated with 100 μg/ml RNase and 20 μg/ml propidium iodide in phosphate-buffered saline (PBS) for 30 min at 37°C and were analyzed using FACSscan (Becton Dickinson, San Jose, CA, USA).

DAPI staining

DAPI staining was performed as previously described (14). Cells were first cultured on 4-chamber slides. After treatment with 100 μM SNP for 12 h, cells were washed twice with PBS and fixed by incubation with 4% paraformaldehyde (PFA) for 30 min. Following washing in PBS, cells were incubated in 1 μg/ml DAPI solution for 30 min in the dark. The cells were analyzed by a fluorescence microscope (Zeiss, Oberkochen, Germany).

TUNEL assay

For *in situ* detection of apoptotic cells, TUNEL assay was performed using the ApoTag[®] peroxidase *in situ* apoptosis detection kit (Boehringer Mannheim GmbH, Mannheim, Germany) (15). C2C12 cells were cultured on 4-chamber slides (Nalge Nunc International, Naperville, IL, USA) at the density of 2 × 10⁴ cells/chamber. After exposure to 100 μM SNP for 12 h, cells were washed with PBS and fixed in 4% PFA for 10 min at 4°C. The fixed cells were then incubated with digoxigenin-conjugated dUTP in the terminal deoxynucleotidyl transferase-catalyzed reaction for 60 min at 37°C in a humidified atmosphere and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with an anti-digoxigenin antibody conjugated with peroxidase for 30 min. The DNA fragments were stained with the peroxidase substrate 3,3'-diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO, USA).

DNA fragmentation assay

For the detection of apoptotic DNA cleavage, DNA fragmentation assay was performed using the Apop-Ladder EXTM DNA fragmentation assay kit (TaKaRa, Shiga) (14). Cells were treated with 100 μM SNP for 12 h and then lysed with 100 μl lysis buffer. The lysate was incubated with 10 μl of 10% sodium dodecyl sulfate (SDS) solution containing 10 μl of Enzyme A at 56°C for 1 h followed by treatment with 10 μl of Enzyme B at 37°C for 1 h. After adding 70 μl of precipitant and

resuspending the resultant pellet in Tris-EDTA (TE) buffer, genomic DNA was visualized by electrophoresis on a 2% agarose gel containing ethidium bromide.

RNA isolation and RT-PCR

To determine the dose- and time-dependent expressions of *p53* and *bax* mRNA, cells were treated with SNP at concentrations of 1, 5, 10, 50, 100, 500, and 1000 μ M for 6, 12, and 24 h, and then the cells were collected. Total RNA was isolated from the cells using RNeasyTM (TEL-TEST, Friendswood, TX, USA) as per the manufacturer's instructions (16). Two microgram of RNA and 2 μ l of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65°C for 10 min. One microliter of AMV reverse transcriptase (Promega), 5 μ l of 10 mM dNTP (Promega), 1 μ l of RNasin (Promega), and 5 μ l of 10 $\times$ AMV RT buffer (Promega) were then added to the mixture, and the final volume was adjusted to 50 μ l with the dimethyl pyrocarbonate (DEPC)-treated water. Subsequently, the reaction mixture was incubated at 42°C for 1 h.

PCR amplification was performed in the 40 μ l reaction volume containing 1 μ l of the appropriate cDNA, 1 μ l of the 10 pM primer set, 4 μ l of 10 $\times$ reaction buffer, 1 μ l of 2.5 mM dNTP (iNtRON, Inc.), and 2 units of *Taq* DNA polymerase (TaKaRa). The sequences of mouse *p53* primers were 5'-GATGACTGCCATG GAGGAGT-3' (a 20-mer sense oligonucleotide) and 5'-CTCGGGTGGCTCATAAGGTA-3' (a 20-mer anti-sense oligonucleotide). The sequences of mouse *bax* primers were 5'-AGATGAACTGGATAGCAATATGGA-3' (a 24-mer sense oligonucleotide) and 5'-CCACCCTG GTCTTGATCCAGACA-3' (a 24-mer anti-sense oligonucleotide). The sequences of the internal control *cyclophilin* primers were 5'-ACCCACCGTGTCT TCGAC-3' (a 20-mer sense oligonucleotide) and 5'-CATTTGCCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide). The expected sizes of the PCR products were 664 bp for *p53*, 270 bp for *bax*, and 291 bp for *cyclophilin*.

The PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: initial denaturation at 94°C for 5 min, followed by 25 amplification cycles, each consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, followed by additional extension step at the end of the procedure at 72°C for 5 min. For *cyclophilin*, the PCR procedure was carried out under the identical conditions except that 25 amplification cycles was performed. The final amount of RT-PCR product was calculated by a densitometer using Molecular AnalystTM version 1.4.1

(Bio-Rad, Hercules, CA, USA).

Western blot analysis

Western blotting for the detection of the p53 protein and its phosphorylation at Thr 18 and bax protein was performed as previously described (16). After treatment with 50 or 100 μ M SNP for 12 h, cells were collected. Cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% NP40, 0.1% SDS, 1 mM PMSF, and 100 μ g/ml leupeptin. Protein concentration was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad). A 50- μ g sample of protein was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Mouse anti-p53 antibody (1:500; Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit anti-phospho p53 (Thr 18) antibody (1:200, Santa Cruz Biotech), and mouse anti-bax antibody (1:1000, Santa Cruz Biotech) were used as the primary antibody. Horseradish peroxidase-conjugated anti-mouse antibodies for p53 and bax (1:1000, Santa Cruz Biotech) were used to probe for p53 and bax, and anti-rabbit antibody for phospho p53 (1:1000, Santa Cruz Biotech) was used as the secondary antibody. The detection of the band was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biothech GmbH, Freiburg, Germany).

Caspase enzyme activity assay

Caspase enzyme activity was measured using the ApoAlert[®] caspase-3 assay kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol (14). In brief, cells were lysed with 50 μ l of chilled Cell Lysis Buffer. A 50- μ l aliquot of 2 $\times$ reaction buffer (containing DTT) and 5 μ l of the appropriate conjugated substrate at a concentration of 1 mM were added to each lysate. The mixture was incubated in a water bath at 37°C for 1 h, and the absorbance was measured with a microtiter plate reader at a test wavelength of 405 nm.

Statistical analyses

The data are expressed as the mean $\pm$ S.E.M. and analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS. The difference was considered statistically significant at $P < 0.05$.

Results

SNP induces NO₂⁻

In the NO detection assay, the nitrite concentration in cells exposed to 50 or 100 μ M SNP for 12 h was increased from 8.37 ± 0.37 (control) to 37.25 ± 1.10 μ M or 54.62 ± 3.14 μ M, respectively (Fig. 1).

Fig. 1. Sodium nitroprusside (SNP) induces nitric oxide (NO) in mouse C2C12 myoblast cells. The results are presented as the mean $\pm$ S.E.M. * represents $P < 0.05$ compared to the control cells. # represents $P < 0.05$ compared to the 50 μ M SNP-treated cells.

The effect of SNP on the viability of C2C12 cells

Figure 2 shows the dose- and time-dependent viability of cells treated with SNP at concentrations of 1, 5, 10, 50, 100, 500, and 1000 μ M for 6, 12, and 24 h. The viabilities of cells incubated with SNP at concentrations of 1, 5, 10, 50, 100, 500, and 1000 μ M for 6 h were $98.79 \pm 4.18\%$, $98.07 \pm 12.91\%$, $91.38 \pm 4.13\%$, $75.49 \pm 7.94\%$, $72.89 \pm 6.87\%$, $72.71 \pm 8.58\%$, and $57.97 \pm 6.10\%$ of the control value, respectively.

The viabilities of cells incubated with SNP at concentrations of 1, 5, 10, 50, 100, 500, and 1000 μ M for 12 h were $94.91 \pm 3.06\%$, $91.64 \pm 3.77\%$, $82.92 \pm 3.22\%$, $70.70 \pm 3.24\%$, $68.73 \pm 2.46\%$, $51.45 \pm 1.01\%$, and $28.61 \pm 0.99\%$ of the control value, respectively.

The viability of cells incubated with SNP at concentrations of 1, 5, 10, 50, 100, 500, and 1000 μ M for 24 h were $82.22 \pm 1.92\%$, $81.08 \pm 1.53\%$, $70.18 \pm 2.08\%$, $60.22 \pm 1.91\%$, $57.82 \pm 1.75\%$, $43.04 \pm 0.98\%$, and

Fig. 2. Dose- and time-dependent effect of sodium nitroprusside (SNP) on mouse C2C12 cell viability. Viabilities were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results are presented as the mean $\pm$ S.E.M. Triangles, Cells were incubated for 6 h; open circles, cells were incubated for 12 h; closed circles, cells were incubated for 24 h. * represents $P < 0.05$ compared to the control cells.

$24.67 \pm 1.74\%$ of the control value, respectively. The cell viability was decreased by SNP treatment dose- and time-dependently.

The change in cell cycle distribution

Flow cytometric analysis revealed that 50 or 100 μ M SNP treatment for 12 h induces the G1 and G2 cell-cycle arrest in C2C12 cells (Fig. 3). The population of cells in the sub-G1 phase in the cells treated with 50 or 100 μ M SNP for 12 h increased from 39.19% (control) to 59.44% or 69.08%, respectively. The population of cells in the G1 phase in cells treated with 50 or 100 μ M SNP for 12 h, on the other hand, decreased from 53.67% (control) to 33.95% or 27.35%, respectively. The population of cells in the G2 phase in the cells treated

Fig. 3. Flow cytometric analysis. The increase in the sub-G1 phase fraction and the decrease in the G1 phase fraction were observed following sodium nitroprusside (SNP) treatment. A, Control cells; B, 50 μ M SNP-treated cells; C, 100 μ M SNP-treated cells.

with 50 or 100 μM SNP for 12 h decreased from 2.56% (control) to 2.04% or 1.23%, respectively.

SNP induces morphological change

To assess the SNP-induced change in cell morphology, cells were examined by a phase-contrast microscopy. As shown in Fig. 4 (upper), cells treated with SNP were detached from the dish, rounded with cytoplasmic blebbing, and irregular in shape.

In DAPI assay, cells were treated with DAPI that specifically stains the nuclei and analyzed by a fluorescence microscopy. The assay revealed the presence of nuclear condensation and perinuclear apoptotic bodies in cells treated with SNP (Fig. 4, middle).

To confirm that SNP induces the apoptosis of C2C12 cells, cells treated with SNP were analyzed in the TUNEL assay. As shown in Fig. 4 (lower), the TUNEL-positive cells (stained dark brown under the light microscope) were observed and the nuclei were condensed.

SNP induces DNA fragmentation

To ascertain that SNP induces apoptosis, DNA fragmentation that reflects the endonuclease activity was analyzed. As shown in Fig. 5, the treatment with 100 μM SNP for 12 h resulted in the formation of

fragments that could be seen as the characteristic ladder pattern by electrophoresis.

The effect of SNP on the expression of p53 and bax mRNA

The RT-PCR analysis of the levels of *p53* and *bax* mRNA was performed to estimate the dose- and time-dependent effect of SNP on the relative levels of these genes. The mRNA level of *p53* and *bax* in the control cells was considered as 1.00.

The levels of *p53* mRNA following the treatment with 50 and 100 μM SNP for 6 h were increased to 1.46 ± 0.26 and 1.54 ± 0.09 , respectively. The levels of *p53* mRNA following the treatment with 50 and 100 μM SNP for 12 h were increased to 1.58 ± 0.01 and 2.32 ± 0.07 , respectively. Finally, the levels of *p53* mRNA following the treatment with 50 and 100 μM SNP for 24 h were increased to 3.40 ± 0.13 and 5.12 ± 0.30 , respectively.

The levels of *bax* mRNA following the treatment with 50 and 100 μM SNP for 6 h were increased to 1.10 ± 0.03 and 1.24 ± 0.06 , respectively. The levels of *bax* mRNA following the treatment with 50 and 100 μM SNP for 12 h were increased to 1.61 ± 0.06 and 2.84 ± 0.33 , respectively. Finally, the levels of *bax* mRNA following the treatment with 50 and 100 μM SNP for 24 h were increased to 3.11 ± 0.19 and 4.06 ± 0.45 , respectively. The levels of *p53* and *bax* mRNA were increased by SNP treatment dose- and time-dependently (Fig. 6).

Western blot analysis of p53 and bax

To test whether *p53* and *bax* are involved in NO-induced apoptosis in myoblast cells, the expression of

Fig. 4. Characterization of the sodium nitroprusside (SNP)-induced cell death in mouse C2C12 cells. Upper: phase-contrast microscopy shows cell shrinkage, irregularity in shape, and cellular detachment in SNP-treated cells. Middle: C2C12 cells stained with 4,6-diamidino-2-phenylindole (DAPI). White arrows indicate the condensed nuclei. Lower: C2C12 cells stained using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. Black arrows show labeled condensed and margined chromatin. The scale bar represents 100 μm .

Fig. 5. Electrophoretic examination of genomic DNA of C2C12 cells. Genomic DNA was extracted and analyzed by electrophoresis on the 2% agarose gel containing ethidium bromide. M, Marker; A, Control cells; B, 100 μM sodium nitroprusside (SNP)-treated cells.

Fig. 6. Dose- and time-dependent effect of sodium nitroprusside (SNP) on mRNA expressions of *p53* and *bax* in mouse C2C12 cells. The levels of *p53* and *bax* mRNA were detected using the reverse transcription-polymerase chain reaction (RT-PCR) analysis. *Cyclophilin* mRNA was used as the internal control. * represents $P < 0.05$ compared to the control cells. # represents $P < 0.05$ compared to the 50 μM sodium nitroprusside (SNP)-treated cells. A, Control cells; B, 50 μM SNP-treated cells; C, 100 μM SNP-treated cells.

Fig. 7. Western blot analysis of the protein level of *p53* and *bax*. The internal control β -actin was detected at the position corresponding to the molecular weight of 46 kDa. A, Control cells; B, 50 μM sodium nitroprusside (SNP)-treated cells; C, 100 μM SNP-treated cells.

p53 protein and its phosphorylation at Thr 18, and the expression of *bax* protein were analyzed by Western blotting.

The expression of *p53* protein (53 kDa) following the treatment with 50 or 100 μM SNP for 12 h was increased in a dose-dependent manner. The expression of *bax* protein (21 kDa) following the treatment with 50 or 100 μM SNP for 12 h was also increased in a dose-dependent manner (Fig. 7). The expression level of phospho-*p53* (Thr 18) in the control cells was consid-

ered as 1.00. The expression of phospho-*p53* (Thr 18) following the treatment with 50 and 100 μM SNP for 12 h was increased to 2.43 ± 0.36 and 3.08 ± 0.55 , respectively (Fig. 8).

Caspase-3 enzyme activity analysis

Caspase-3 enzyme activity was measured using DEVD-peptide-nitroanilide (*pNA*). After incubation with 100 μM SNP for 12 h, the amount of DEVD-*pNA* cleaved was significantly increased from 5.50 ± 0.73 (control value) to 11.59 ± 2.11 pM (Fig. 9).

Discussion

NO is implicated in the modulation of apoptotic cell death (17, 18). However, the effect of NO is different depending on the NO concentration. The low to moderate concentration of NO is known to exert the protective effect against oxidative stress, damage, and cell death (19, 20). In contrast, the high concentration of NO induces apoptotic cell death in various cell types such as PC12 cells, myocytes, smooth muscle cells, and PGT- β immortalized pineal cells (21–23).

It has been reported that aging, several muscle-wast-

Fig. 8. Results of Western blot analysis of expression level of phospho-p53 (Thr 18) in mouse C2C12 cells. The expression level of β -actin protein was used as the internal control. * represents $P < 0.05$ compared to the control cells. # represents $P < 0.05$ compared to the 50 μ M sodium nitroprusside (SNP)-treated cells. A, Control cells; B, 50 μ M SNP-treated cells; C, 100 μ M SNP-treated cells.

Fig. 9. The effect of sodium nitroprusside (SNP) on the caspase-3 enzyme activity. The rate of DEVD-pNA cleavage was measured at a wavelength of 405 nm. * represents $P < 0.05$ compared to the control cells. A, Control cells; B, 100 μ M SNP-treated cells; C, SNP-treated cells with DEVD-fmk added. DEVD-fmk is a caspase-3 inhibitor.

ing diseases, and intense muscle contraction increase NO production in the skeletal muscles and excessive production of NO induces oxidative stress and cell death in muscles (2, 24). Here, we investigated whether NO induces apoptotic cell death in mouse C2C12 myoblasts.

Nitrite is the stable end product of NO that indicates NO production in cells (25, 26). Our data showed that

the nitrite concentration in the C2C12 myoblast was increased by SNP treatment in a dose-dependent manner. We also observed that cell viability was significantly decreased by SNP treatment in a dose-dependent manner, showing that SNP exerts a cytotoxic effect in C2C12 myoblasts. From the data obtained in the flow cytometric analysis of DNA contents, the increase in the fraction of cells in the sub-G1 phase was observed in SNP-treated cells.

In this study, NO induced the apoptotic changes in C2C12 cells. Under the phase-contrast microscope, SNP-treated cells showed cell shrinkage, cytoplasmic condensation, and irregularity in shape. Apoptotic bodies were detected in SNP-treated cells stained with DAPI. In addition, TUNEL-positive cells, indicative of apoptotic DNA strand breaks and nicks in the DNA molecules, were also detected in the SNP-treated cells.

It is known that apoptosis involves the activation of endonucleases resulting in the cleavage of genomic DNA into well-defined fragments that appear as the characteristic ladder pattern upon agarose gel electrophoresis (27). To provide evidence supporting the involvement of apoptosis in the SNP-induced cytotoxicity, the DNA fragmentation assay was performed. In this study, the distinctive ladder pattern characteristic of apoptotic cell death was detected in cells treated with SNP.

Apoptosis is regulated by the complex interplay between regulatory proteins. The molecular mechanism underlying the NO-mediated apoptosis involves different pathways depending on the cell type. It is well known that NO increases the expression of p53 leading to apoptosis in various cells. Bax, a death-promoting gene, is required for the NO-mediated cell death (28). Xiang et al. demonstrated that the activation of p53 regulates the expression of bax, a p53-responsive gene (9). Bax mediates the p53-dependent apoptosis in neuronal cells. The present results show that SNP may induce apoptotic cell death by increasing the expression of p53 and bax in C2C12 myoblast cells.

Caspases, a family of cysteine proteases, are an integral part of the apoptotic pathway. Numerous studies have also shown that NO-induced apoptotic cell death occurs through a caspase-independent pathway in various cell types (29–31). Uchiyama et al. (32) reported that caspase activation plays a crucial role in cardiomyocyte apoptosis induced by exposure to an NO donor, *S*-nitro-*N*-acetyl-penicillamine (SNAP). The present results show that SNP may induce apoptotic cell death by increasing the enzyme activity of caspase-3 in C2C12 myoblast cells.

In conclusion, we have shown that NO may induce apoptotic cell death in myoblast cells through the acti-

vation of the p53-, bax-, and caspase-dependent intracellular death-related pathway. For better understating of the underlying mechanism of the NO-induced skeletal muscle cell death, however, the detailed signal pathway of NO controlling the expression of apoptosis-associated genes in myoblast cells needs to be further clarified.

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