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Nitric Oxide From Both Exogenous and Endogenous Sources Activates Mitochondria-Dependent Events and Induces Insults to Human Chondrocytes

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Abstract During inflammation, overproduction of nitric oxide (NO) can damage chondrocytes. In this study, we separately evaluated the toxic effects of exogenous and endogenous NO on human chondrocytes and their possible mechanisms. Human chondrocytes were exposed to sodium nitroprusside (SNP), an NO donor, or a combination of lipopolysaccharide (LPS) and interferon-γ (IFN-γ) as the exogenous and endogenous sources of NO, respectively. Administration of SNP or a combination of LPS and IFN-γ in human chondrocytes increased cellular NO levels but decreased cell viability. Exposure to exogenous or endogenous NO significantly induced apoptosis of human chondrocytes. When treated with exogenous or endogenous NO, the mitochondrial membrane potential time-dependently decreased. Exposure to exogenous or endogenous NO significantly enhanced cellular reactive oxygen species (ROS) and cytochrome *c* (Cyt *c*) levels. Administration of exogenous or endogenous NO increased caspase-3 activity and consequently induced DNA fragmentation. Suppression of caspase-3 activation by Z-DEVD-FMK decreased NO-induced DNA fragmentation and cell apoptosis. Similar to SNP, exposure of human chondrocytes to S-nitrosoglutathione (GSNO), another NO donor, caused significant increases in Cyt *c* levels, caspase-3 activity, and DNA fragmentation, and induced cell apoptosis. Pretreatment with N-monomethyl arginine (NMMA), an inhibitor of NO synthase, significantly decreased cellular NO levels, and lowered endogenous NO-induced alterations in cellular Cyt *c* amounts, caspase-3 activity, DNA fragmentation, and cell apoptosis. Results of this study show that NO from exogenous and endogenous sources can induce apoptotic insults to human chondrocytes via a mitochondria-dependent mechanism. J. Cell. Biochem. 101: 1520–1531, 2007.

Key words: human chondrocytes; nitric oxide; apoptosis; mitochondrial dysfunction; apoptotic factors; caspase activation

Chondrocytes are responsible for preventing cartilage degeneration through maintaining the extracellular matrix [van der Kraan et al., 2002]. In cartilage subjected to trauma, chondrocytes may be damaged or even die [Quinn et al., 2001; Redman et al., 2004]. Studies on human osteoarthritic cartilage have revealed a direct correlation between disease severity and the degree of chondrocyte damage [Aigner and Kim, 2002; Murray et al., 2004]. Thus, chondrocyte insults are a critical factor in inducing joint degeneration. A variety of inflammatory cytokines and reactive oxygen species (ROS) contribute to the pathophysiological regulation of chondrocytes [Mathy-Hartert et al., 2002; Kuhn et al., 2003]. Nitric oxide (NO), one of the ROS, has been implicated in the modulation of cartilage remodeling [Cake

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et al., 2005]. In untreated chondrocytes, NO can be constitutively produced [Fermor et al., 2005]. Meanwhile, after stimulation of inflammatory cytokines or mechanical stress, NO may be overproduced by chondrocytes and induce insults to cells [Blanco et al., 1995; Kuhn et al., 2004; Shan et al., 2004]. However, the molecular mechanism of NO-induced chondrocyte injuries still needs to be elucidated.

Apoptosis of chondrocytes has been implicated as playing a crucial role in morphogenetic, histogenetic, and phylogenetic processes of cartilage tissue development [Perez et al., 2005]. There are many intrinsic and extrinsic factors involved in apoptosis regulation [Nicholson and Thornberry, 1997; Chung et al., 2001]. NO can be a death regulator which induces cell apoptosis [Chung et al., 2001]. Mitochondria can regulate the process of cell apoptosis [Yu et al., 2002]. After stimulation, depolarization of the mitochondrial membrane leads to mitochondrial dysfunction, release of apoptotic factors from mitochondria to the cytoplasm, and cell apoptosis [Hortelano et al., 1999; Blom et al., 2003]. Intracellular ROS and cytochrome *c* (Cyt *c*) are two typical mitochondria-related apoptotic factors. Following mitochondrial dysfunction, the release of intracellular ROS increases cellular oxidative stress and induces cell damage [Fleury et al., 2002; Kuhn et al., 2004]. Depolarization of the mitochondrial membrane is usually accompanied by an increase in Cyt *c* release [Hortelano et al., 1999]. Sequentially, Cyt *c* release can further trigger caspase activation and digestion of key proteins, ultimately driving cells to undergo apoptosis [Goyal, 2001; Chang et al., 2006]. A previous study showed that NO can damage chondrocytes through selectively suppressing mitochondrial complex IV enzyme activity and the respiratory chain reaction [Maneiro et al., 2005].

During inflammation, NO produced by chondrocytes themselves or neighboring cells, including immune cells, can induce chondrocyte injuries [Mathy-Hartert et al., 2002; Kuhn et al., 2003]. However, the detailed signal-transducing mechanism of NO-induced chondrocyte apoptosis is still not well known. Investigating NO-induced chondrocyte insults is crucial to the clinical treatment of cartilage dysfunction. Therefore, in this study, we attempted to evaluate the damaging effects of exogenous and endogenous NO on human chondrocytes and the possible molecular mechanisms.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

Human chondrocytes purchased from Cell Applications (San Diego, CA, USA) were derived from normal human articular cartilage and can be cultured through at least 10 population doublings. The cells were seeded in chondrocyte growth medium (Cell Applications), which is fully supplemented for culturing and propagating chondrocytes. The cells were seeded in 75-cm^2 flasks at 37 °C in a humidified atmosphere of 5% CO_2 and grown to confluence prior to drug treatment.

Sodium nitroprusside (SNP) and S-nitrosoglutathione (GSNO), purchased from Sigma (St. Louis, MO, USA), were freshly dissolved in phosphate-buffered saline (PBS) buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). Human chondrocytes were exposed to SNP or GSNO as the exogenous source of NO. As the endogenous source of cellular NO, human chondrocytes were treated with a combination of 1 μg/ml lipopolysaccharide (LPS) and 100 IU/ml interferon (IFN)-γ. To inhibit endogenous NO synthesis, human chondrocytes were pretreated with 1 mM N-monomethyl arginine (NMMA) for 1 h, and then exposed to a combination of LPS and IFN-γ.

Quantification of Cellular Nitric Oxide Levels

Levels of cellular NO in human chondrocytes were determined according to the technical bulletin of a Bioxytech NO assay kit (OXIS International, Portland, OR, USA) as described previously [Chang et al., 2006]. In this kit, nitrate reductase is provided to reduce nitrate to nitrite so that total nitrite in the culture medium is detected. After drug administration, the culture medium of human chondrocytes was collected and centrifuged. The supernatant fractions were reacted with nitrate reductase. Following a reaction of the supernatant with sulfanilamide and N-1-napthylethylenediamine, a colorimetric azo compound formed and was quantified using an Anthos 2010 microplate photometer (Anthos Labtec Instruments, Lagerhausstrasse, Wals/Salzburg, Austria).

Analysis of Lactate Dehydrogenase

Amounts of lactate dehydrogenase released from human chondrocytes into the culture medium were measured for evaluating the cytotoxicity of NO to cells [Chen et al., 2003]. Human chondrocytes (1×10^5 cells) were seeded in 24-well tissue culture plates (Corning Costar Corporation, Cambridge, MA, USA). After drug administration, the culture medium was collected and centrifuged. Amounts of lactate dehydrogenase in the supernatants were analyzed using a model 7450 automatic auto-analyzer system from Hitachi (Tokyo, Japan).

Assay of Cell Membrane Integrity

The integrity of cellular membranes was determined by a trypan blue exclusion method to further evaluate NO's cytotoxicity to human chondrocytes [Chen et al., 2002]. Briefly, human chondrocytes (2×10^5 cells) were cultured in 24-well tissue culture plates. After drug treatment, human chondrocytes were trypsinized by 0.1% trypsine-EDTA (Gibco, BRL, Grand Island, NY, USA). Following centrifuging and washing, human chondrocytes were suspended in 1× PBS buffer and stained with an equal volume of trypan blue dye. The fractions of damaged cells with a blue signal were counted using a reverse-phase microscope.

Analysis of DNA Fragmentation

DNA fragmentation in human chondrocytes was quantified to evaluate if exogenous or endogenous NO damages nuclear DNA as described previously [Chen et al., 2005b]. The BrdU-labeled histone-associated DNA fragments in the cytoplasm of cell lysates were detected according to the instructions of the cellular DNA fragmentation enzyme-linked immunosorbent assay kit (Boehringer Mannheim, Indianapolis, IN, USA). Briefly, human chondrocytes (2×10^5) were subcultured in 24-well tissue culture plates and labeled with BrdU overnight. Cells were harvested and suspended in the culture medium. One hundred microliters of cell suspension was added to each well of 96-well tissue culture plates. Human chondrocytes were cocultured with SNP for another 8 h at 37°C in a humidified atmosphere of 5% CO_2. Amounts of BrdU-labeled DNA in the cytoplasm were quantified using an Anthos 2010 microplate photometer (Anthos Labtec Instruments) at a wavelength of 450 nm.

Quantification of Apoptotic Cells

The proportion of human chondrocytes undergoing apoptosis was determined by detecting cells which were arrested at the sub-G1 stage according to a previously described method [Chen et al., 2002]. After drug treatment, human chondrocytes were harvested and fixed in cold 80% ethanol. Following 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 Jose, CA, USA) on the basis of a 560-nm dichromic mirror and a 600-nm bandpass filter.

Quantification of the Mitochondrial Membrane Potential

The membrane potential of mitochondria in human chondrocytes was determined according to a previously described method [Chen et al., 2005a]. Briefly, human chondrocytes (5×10^5 cells) were seeded in 12-well tissue culture plates overnight. After drug administration, human chondrocytes were harvested and incubated with 3,3′-dihexyloxacarbocyanine ($DiOC_6$), 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 in 1× PBS buffer. The fluorescence intensities in human chondrocytes were analyzed by flow cytometry (FACS Calibur).

Determination of Intracellular Reactive Oxygen Species

Levels of intracellular ROS were quantified following a previously described method [Chen et al., 2005a]. Briefly, 5×10^5 human chondrocytes were cultured in 12-well tissue culture plates overnight, and then cotreated with SNP and 2′,7′-dichlorofluorescin diacetate (DCFH-DA), an ROS-sensitive dye. After SNP treatment, human chondrocytes were harvested and suspended in 1× PBS buffer. The relative fluorescence intensities in human chondrocytes were quantified by flow cytometry (FACS Calibur).

Immunoblot Analysis of Cellular Cytochrome c Levels

After drug administration, cell lysates were collected after dissolving cells in 50 μl of ice-cold radioimmunoprecipitation assay buffer (25 mM Tris-HCl (pH 7.2), 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, and

1 mM EDTA). Protein concentrations were quantified using a bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Cytosolic proteins (100 μg per well) were subjected to 12% polyacrylamide gels, and electrophoretically blotted onto nitrocellulose membranes. Cyt *c* protein was immunodetected using a mouse monoclonal antibody against rat Cyt *c* protein (Transduction Laboratories, Lexington, KY, USA). β-actin was immunodetected by a mouse monoclonal antibody against mouse β-actin (Sigma) as an internal control. Intensities of these immunoreactive protein bands were determined using a UVIDOCMW version 99.03 digital imaging system (UVtec, Cambridge, UK).

Fluorogenic Substrate Assay for Caspase-3 Activities

The enzyme activity of caspase-3 was assayed using a fluorometric assay kit (R&D Systems, Minneapolis, MN, USA). Briefly, after drug treatment, human chondrocytes were lysed using a buffer containing 1% Nonidet P-40, 200 mM NaCl, 20 mM Tris/HCl (pH 7.4), 10 μg/ml leupeptin, 0.27 U/ml aprotinin, and 100 μm PMSF. The cell extracts (25 μg total protein) were incubated with 50 μM of a specific fluorogenic peptide substrate (DEVD) in 200 μl of a cell-free system buffer comprised of 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 1 mM dithiothreitol. Intensities of the fluorescent products were measured using an LS 55 spectrometer of PerkinElmer Instruments (Shelton, CT, USA). To determine the effects of caspase-3 activation on DNA fragmentation and cell apoptosis, human chondrocytes were pretreated with 50 μM Z-DEVD-FMK, an inhibitor of caspase-3, for 1 h, and then exposed to exogenous or endogenous NO.

Statistical Analysis

The statistical difference between the control and drug-treated groups was considered significant when the *P*-value of Duncan's multiple range test was <0.05. Statistical analysis between groups over time was carried out using two-way ANOVA.

RESULTS

To evaluate the toxicity of exogenous NO to human chondrocytes, cells were treated with SNP, and cellular NO levels, lactate dehydrogenase release, and the membrane integrity were determined (Tables I,II). Administration of 0.1, 0.5, and 1 mM SNP to human chondrocytes for 24 h significantly increased cellular NO levels by 38%, and 2.8- and 5.6-fold, respectively (Table I). When the concentrations reached 0.5 and 1 mM, SNP significantly increased the lactate dehydrogenase levels in the culture medium by 85% and 2.5-fold, respectively. Exposure to 0.05 and 0.1 mM SNP significantly decreased cell viability by 37 and 57%, respectively (Table I). Administration of 1 mM SNP to human chondrocytes for 1, 6, 12, and 24 h enhanced cellular NO levels by 71%, and 2.5-, 5.3-, and 6.3-fold, respectively (Table II). The release of lactate dehydrogenase was augmented by 57, 83%, and 2.6-fold after treatment with SNP for 6, 12, and 24 h,

TABLE I. Concentration-Dependent Effects of Sodium Nitroprusside on Cellular Nitric Oxide Levels, Lactate Dehydrogenase Amounts, and Cell Viability

SNP (mM)	NO (μM)	LDH (U/L)	Cell viability (cell number × 10^3)
0	2.9 ± 0.2	26 ± 6	143 ± 29
0.05	3.5 ± 0.4	28 ± 7	145 ± 33
0.1	4.0 ± 0.3*	32 ± 9	158 ± 37
0.5	8.1 ± 0.5*	48 ± 11*	89 ± 18*
1.0	16.2 ± 0.7*	65 ± 13*	61 ± 14*

Human chondrocytes were exposed to 0.05, 0.1, 0.5, and 1 mM SNP for 24 h. Cellular NO levels were quantified by the Griess reaction. Amounts of lactate dehydrogenase in the culture medium were determined by an autoanalyzer as described in 'Materials and Methods'. Cell viability was assayed by the trypan blue exclusion method. Each value represents the mean ± SEM for n = 6.
*Values significantly differ from the respective control, $P < 0.05$.

TABLE II. Time-Dependent Effects of Sodium Nitroprusside on Cellular Nitric Oxide Levels, Lactate Dehydrogenase Amounts, and Cell Viability

Time (h)	NO (μM)	LDH (U/L)	Cell viability (cell number × 10^3)
0	3.1 ± 0.3	23 ± 4	151 ± 32
1	5.3 ± 0.4*	28 ± 6	164 ± 27
6	7.9 ± 0.9*	36 ± 4*	93 ± 21*
12	16.5 ± 2.1*	42 ± 7*	72 ± 19*
24	19.4 ± 3.5*	59 ± 8*	56 ± 8*

Human chondrocytes were exposed to 1 mM SNP for 1, 6, 12, and 24 h. Cellular NO levels were quantified by the Griess reaction. Amounts of lactate dehydrogenase in the culture medium were determined by an autoanalyzer as described in Materials and Methods'. Cell viability was assayed by the trypan blue exclusion method. Each value represents the mean ± SEM for n = 12.
*Values significantly differ from the respective control, $P < 0.05$.

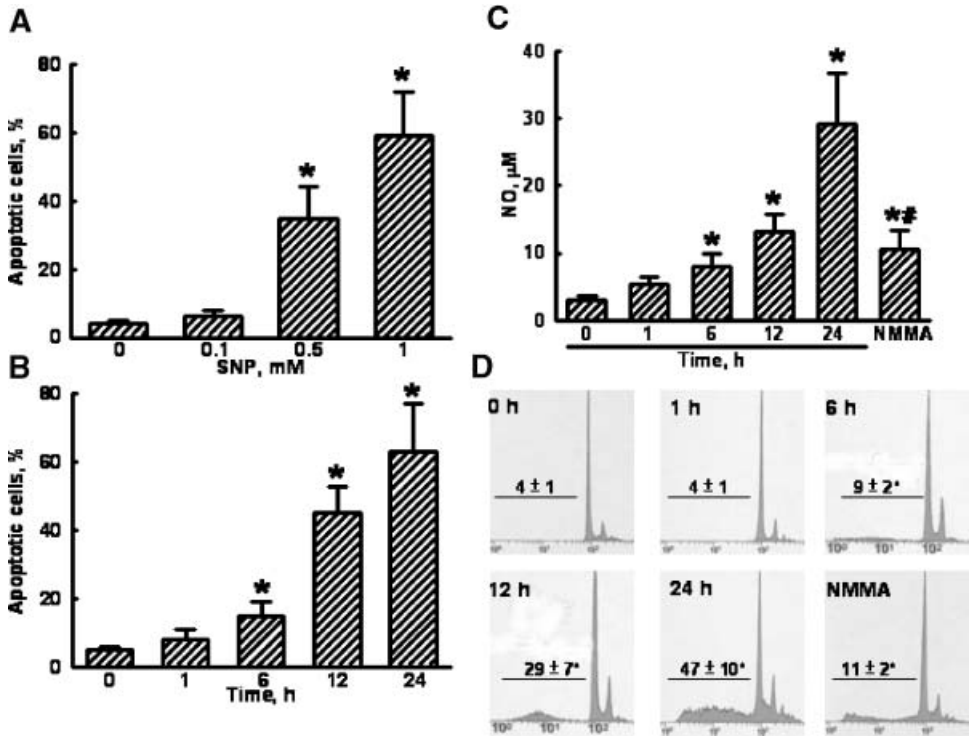

Fig. 1. Respective effects of exogenous and endogenous NO on apoptotic insults. Human chondrocytes were exposed to SNP or a combination of LPS and IFN-γ as the exogenous and endogenous NO sources, respectively. Apoptotic cells were analyzed using flow cytometry after exposure to 0.1, 0.5, and 1 mM SNP for 24 h (**A**) or to 1 mM SNP for 1, 6, 12, and 24 h (**B**). Following administration of a combination of LPS and IFN-γ or NMMA, cellular NO levels were quantified using the Griess reaction (**C**), and the proportion of apoptotic cells was determined (**D**). Each value represents the mean ± SEM for n = 12. The symbols ''*'' and ''#'' indicate that a value significantly ($P < 0.05$) differs from the respective control and 24-h treated groups, respectively.

respectively (Table II). Exposure to SNP for 6, 12, and 24 h, respectively, caused significant 38, 52, and 63% decreases in cell viability (Table II).

An apoptotic analysis was carried out to determine the death mechanism induced by exogenous and endogenous NO (Fig. 1). Exposure of human chondrocytes to 0.5 and 1 mM SNP for 24 h significantly induced cell apoptosis by 35 and 59% (Fig. 1A). After treatment for 6, 12, and 24 h, SNP, respectively, induced cell apoptosis by 15, 45, and 63% (Fig. 1B). Administration of a combination of LPS and IFN-γ in human chondrocytes for 6, 12, and 24 h significantly enhanced the amounts of cellular NO by 2.5-, 4.4-, and 9.4-fold, respectively (Fig. 1C). Pretreatment with NMMA significantly ameliorated 64% of the cellular NO levels induced by LPS and IFN-γ. In parallel with the increases in endogenous NO, administration of LPS and IFN-γ for 6, 12, and 24 h, respectively, induced cell apoptosis by 9, 29, and 47% (Fig. 1D). NMMA significantly lowered the proportions of apoptotic cells by 77%.

To further investigate the roles of mitochondria in NO-induced apoptotic insults to human chondrocytes, the mitochondrial membrane potential was quantified (Fig. 2). Administration of 0.5 and 1 mM SNP to human chondrocytes for 24 h significantly decreased the mitochondrial membrane potential by 24 and 45%, respectively (Fig. 2A). After exposure to 1 mM SNP for 1, 6, 12, and 24 h, the mitochondrial membrane potentials of human chondrocytes were suppressed by 16, 26, 34, and 48%, respectively (Fig. 2B). Administration of a combination of LPS and IFN-γ for 6, 12, and 24 h, respectively, decreased the mitochondrial membrane potential by 18, 25, and 32% (Fig. 2C).

Intracellular ROS levels and cellular Cyt *c* release were analyzed to evaluate if these two mitochondria-related apoptotic factors are involved in NO-induced cell apoptosis (Figs. 3,4). Exposure of human chondrocytes to 0.1, 0.5, and 1 mM SNP, respectively, caused significant 57%, and 2.7- and 4-fold increases in intracellular ROS levels (Fig. 3A). After

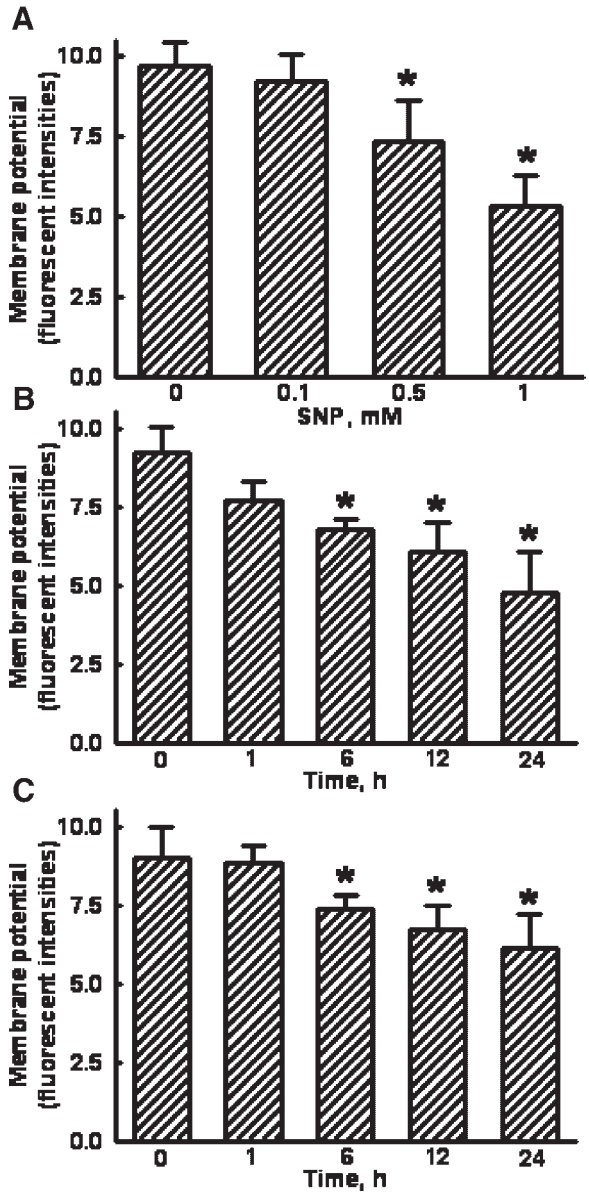

Fig. 2. Respective effects of exogenous and endogenous NO on the mitochondrial membrane potential. Human chondrocytes were exposed to SNP and a combination of LPS and IFN-γ as the exogenous and endogenous sources, respectively. The mitochondrial membrane potential was determined by staining with a cationic dye, DiOC6, and quantified using flow cytometry after exposure to 0.1, 0.5, and 1 mM SNP for 24 h (**A**) or to 1 mM SNP for 1, 6, 12, and 24 h (**B**). The effects of endogenous NO on the mitochondrial membrane potential were also evaluated (**C**). Each value represents the mean ± SEM for n = 6. ''*'' Values significantly differ from the respective control, $P < 0.05$.

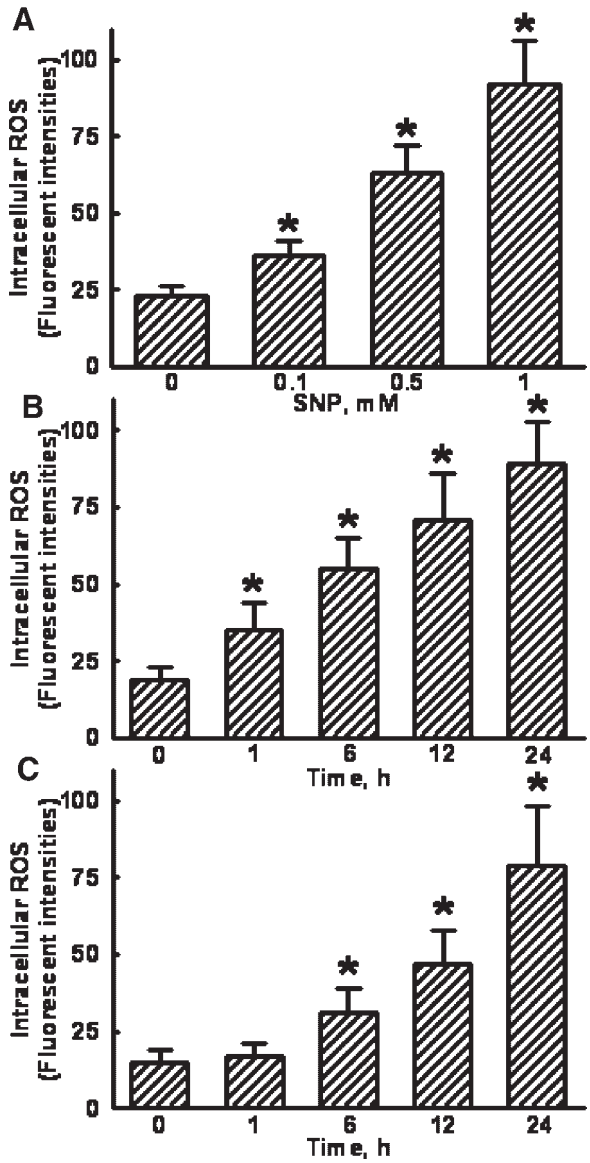

Fig. 3. Respective effects of exogenous and endogenous NO on the levels of intracellular ROS. Human chondrocytes were exposed to SNP and a combination of LPS and IFN-γ as the exogenous and endogenous sources, respectively. Levels of intracellular ROS were revealed by a ROS-sensitive dye, DCFH-DA, and quantified using flow cytometry after exposure to 0.1, 0.5, and 1 mM SNP for 24 h (**A**) or to 1 mM SNP for 1, 6, 12, and 24 h (**B**). The effects of endogenous NO on intracellular ROS were also evaluated (**C**). Each value represents the mean ± SEM for n = 6. ''*'' Values significantly differ from the respective control, $P < 0.05$.

administration of 1 mM SNP for 1, 6, 12, and 24 h, intracellular ROS levels were, respectively, augmented by 84%, and 2.9-, 3.7-, and 4.7-fold (Fig. 3B). Treatment of human chondrocytes with LPS and IFN-γ for 6, 12, and 24 h, respectively, enhanced the amounts of intracellular ROS by 2.1-, 3.1-, and 5.3-fold (Fig. 3C).

Exposure of human chondrocytes to 1 mM SNP for 6, 12, and 24 h increased the levels of Cyt *c* (Fig. 4A, top panel, lanes 3–6). After

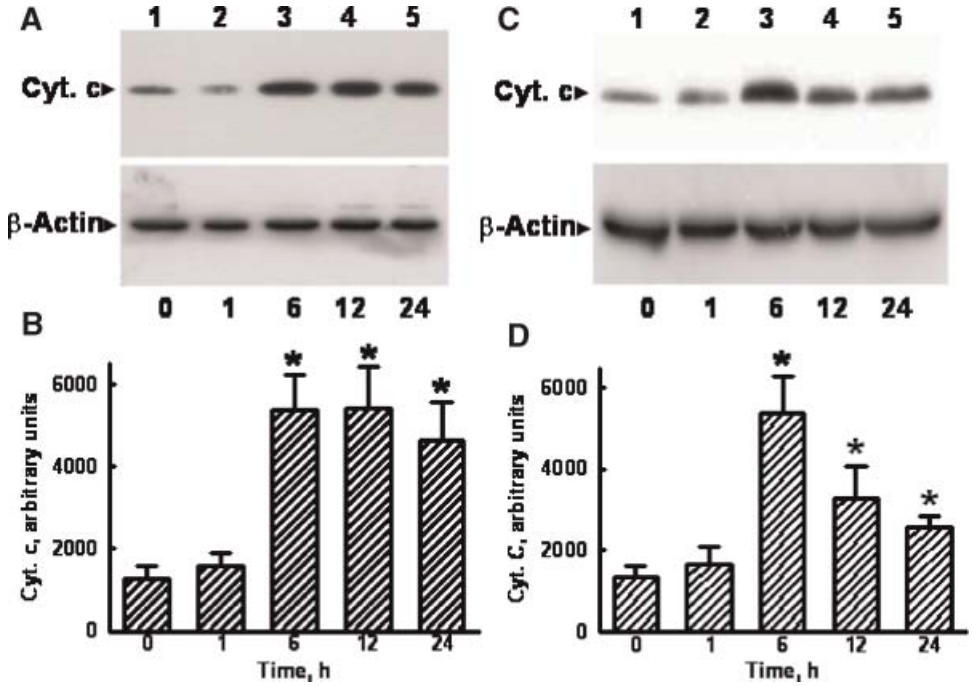

Fig. 4. Respective effects of exogenous and endogenous NO on cellular Cyt *c* protein levels. Human chondrocytes were exposed to SNP and a combination of LPS and IFN-γ as the exogenous and endogenous sources, respectively. Human chondrocytes were exposed to 1 mM SNP (**A**,**B**) or to a combination of LPS and IFN-γ (**C**,**D**) for 1, 6, 12, and 24 h. After preparation of cytosolic proteins, these proteins were subjected to SDS–PAGE and blotted onto nitrocellulose membranes. Cyt *c* was immunodetected using a monoclonal antibody against rat Cyt *c* protein (A,C). β-actin was detected as the internal standard. Intensities of these immunorelated protein bands were quantified by a digital system (B,D). Each value represents the mean ± SEM for n = 4. ''*'' Values significantly differ from the respective control, $P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

administration of LPS and IFN-γ for 6, 12, and 24 h, the levels of Cyt *c* in human chondrocytes were obviously augmented (Fig. 4C, top panel, lanes 3–6). β-actin was immunodetected as the internal standard (Fig. 4A,C, bottom panel). These protein bands were quantified and analyzed (Fig. 4B,D). Administration of 1 mM SNP for 6, 12, and 24 h significantly increased cellular Cyt *c* levels by 4.2-, 4.3-, and 3.6-fold, respectively (Fig. 4B). After treatment with LPS and IFN-γ for 6, 12, and 24 h, the amounts of Cyt *c* were, respectively, augmented by 4.1- and 2.5-fold, and 95% (Fig. 4D).

Caspase-3 activity and DNA fragmentation were assayed to determine the possible cascade mechanism of NO-induced cell apoptosis (Fig. 5). Administration of SNP for 6, 12, and 24 h significantly increased caspase-3 activities by 50%, 2-fold, and 73%, respectively (Fig. 5A). Caspase-3 activity was, respectively, enhanced by 2.1-fold, 69 and 78% following treatment with LPS and IFN-γ for 6, 12, and 24 h. Pretreatment with Z-DEVD-FMK, a caspase-3 inhibitor, significantly lowered the endogenous and exogenous NO-induced increases in caspase-3 activities by 46 and 49%, respectively (Fig. 5A). Exposure to SNP for 6, 12, and 24 h significantly induced DNA fragmentation by 83%, and 2.3- and 3.6-fold, respectively (Fig. 5B). Treatment with LPS and IFN-γ for 6, 12, and 24 h, respectively, induced DNA fragmentation by 73%, and 2.3- and 2.5-fold. When pretreated with Z-DEVD-FMK, exogenous and endogenous NO-induced DNA fragmentation significantly decreased by 42 and 39%, respectively (Fig. 5B). The exogenous and endogenous NO-induced apoptosis of human chondrocytes was significantly ameliorated after pretreatment with Z-DEVD-FMK by 65 and 64%, respectively (Fig. 5C).

The effects of GSNO on insults to human chondrocytes were evaluated (Table III). Exposure to GSNO significantly increased cellular NO and Cyt *c* levels by 6.2- and 2-fold, respectively. When administered with GSNO, caspase-3 activity was enhanced 2.7-fold. Treatment with GSNO caused a significant twofold increase in DNA fragmentation, and increased cell apoptosis by 51% (Table III).

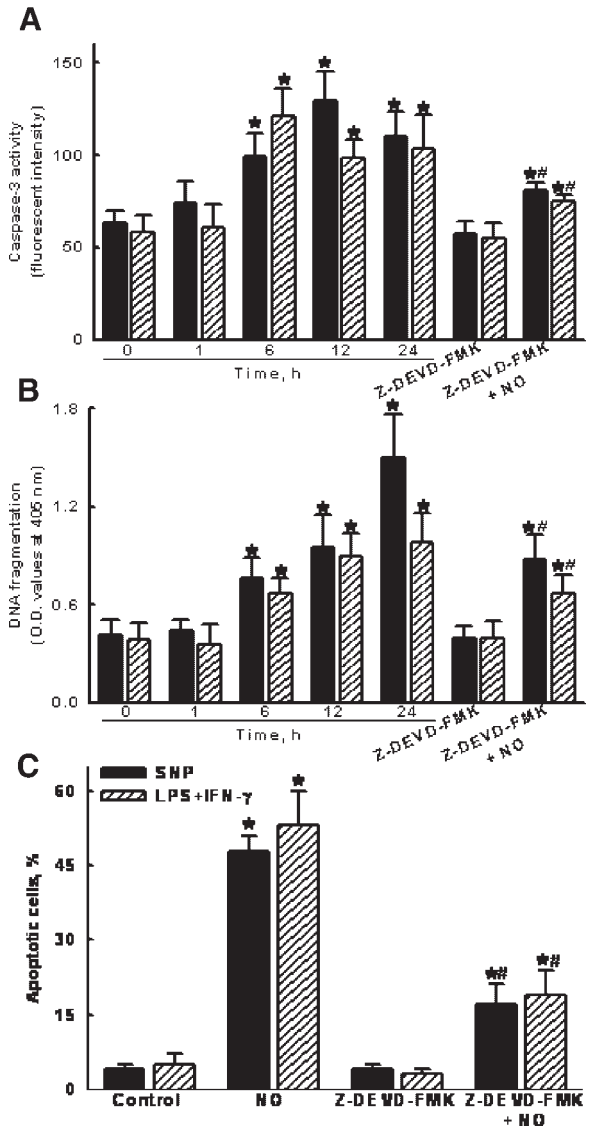

Fig. 5. Respective effects of exogenous and endogenous NO on caspase-3 activity and DNA fragmentation. Human chondrocytes were exposed to SNP and a combination of LPS and IFN-γ as the exogenous and endogenous sources, respectively. Human chondrocytes were exposed to 1 mM SNP or to a combination of LPS and IFN-γ for 1, 6, 12, and 24 h. To determine the effects of caspase-3 activation on DNA fragmentation and cell apoptosis, human chondrocytes were pretreated with 50 μM Z-DEVD-FMK, an inhibitor of caspase-3, for 1 h, and then separately exposed to exogenous and endogenous NO. The activity of caspase-3 was analyzed by a fluorogenic substrate assay (**A**). DNA fragmentation was assayed using an ELISA kit (**B**). Apoptotic cells were quantified using flow cytometry (**C**). Each value represents the mean ± SEM for n = 6. The symbols "*" and "#" indicate that a value significantly ($P < 0.05$) differs from the respective control and NO-treated groups, respectively.

To further determine the effects of suppression of endogenous NO synthesis on activities of human chondrocytes, NMMA was administered to cells (Fig. 6). Exposure to LPS and IFN-γ increased cellular Cyt *c* levels (Fig. 6A, top panel, lane 2). Pretreatment with NMMA decreased the amounts of Cyt *c* induced by LPS and IFN-γ (lane 4). β-actin was immunodetected as the internal standard (Fig. 6A, bottom panel). These protein bands were quantified and analyzed (Fig. 6B). After exposure to LPS and IFN-γ, Cyt *c* was enhanced by 3.1-fold. Pretreatment with NMMA significantly lowered endogenous NO-induced increases in Cyt *c* (Fig. 6B). Administration of LPS and IFN-γ increased caspase-3 activity and DNA fragmentation by 2.4- and 2.3-fold, respectively (Fig. 6C,D). Following pretreatment with NMMA, the endogenous NO-induced alteration in caspase-3 activity and DNA fragmentation significantly decreased.

TABLE III. Effects of S-Nitrosoglutathione on Cellular Nitric Oxide Levels, Cyt *c* Amounts, Caspase-3 Activity, DNA Fragmentation, and Cell Apoptosis

	Control	GSNO
NO, μM	3.1 ± 0.4	19.3 ± 4.6*
Cyt *c*, arbitrary units	1487 ± 228	3015 ± 475*
Caspase-3 activity, fluorescence intensities	51 ± 14	138 ± 26*
DNA fragmentation, O.D. values at 450 nm	0.487 ± 0.098	0.976 ± 0.109*
Apoptotic cells, %	4 ± 1	51 ± 11*

Human chondrocytes were exposed to 1 mM GSNO. Cellular NO levels were quantified by the Griess reaction. Amounts of Cyt. *c* were analyzed by an immunoblot. Caspase-3 activity was detected by a fluorogenic substrate assay using DEVD as the substrate. DNA fragmentation was assayed using an ELISA kit. Apoptotic cells were quantified using flow cytometry. Each value represents the mean ± SEM for n = 3.
*Values significantly differ from the respective control, $P < 0.05$.

DISCUSSION

This study provides in vitro data to show that overproduction of NO from exogenous or endogenous sources can induce apoptotic insults to human chondrocytes via a mitochondria-dependent mechanism. After exposure to SNP or a combination of LPS and IFN-γ, cellular NO levels were significantly enhanced. Increases in cellular NO levels simultaneously induced cell death via an apoptotic mechanism. Sequentially, overproduction of exogenous or endogenous NO caused suppression of the mitochondrial membrane potential, increases in intracellular ROS and Cyt *c* release, caspase-3 activation, and DNA fragmentation. Similarly, exposure to GSNO induced insults to

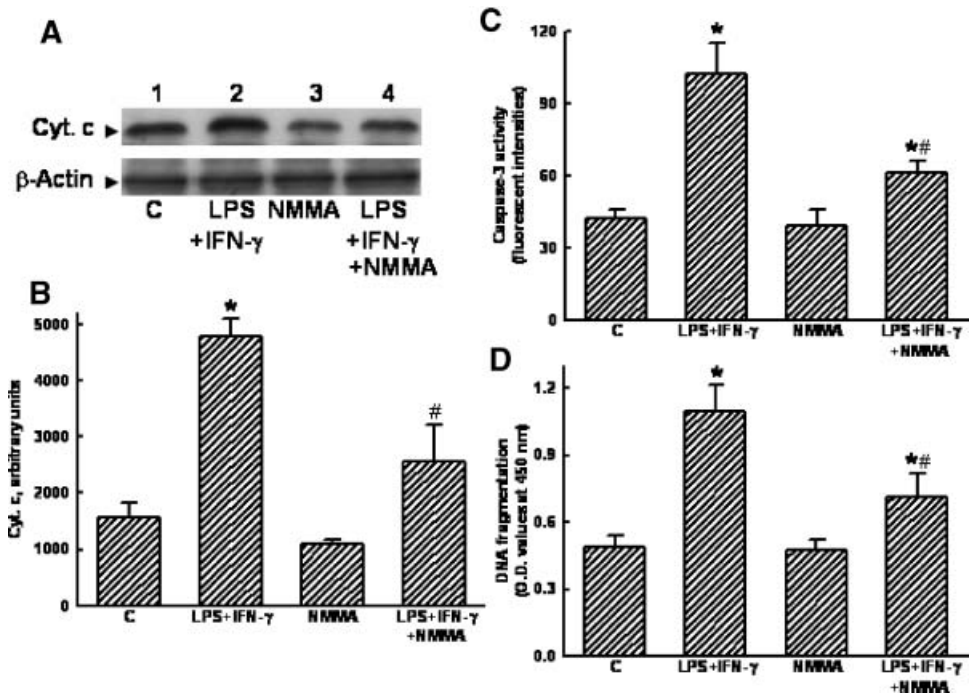

Fig. 6. Effects of NMMA on cellular Cyt *c* levels, caspase-3 activity, and DNA fragmentation. Human chondrocytes were pretreated with 1 mM NMMA for 1 h, and then exposed to a combination of LPS and IFN-γ. Cyt *c* was immuno-detected and quantified (**A,B**). The activity of caspase-3 was assayed by a fluorogenic substrate assay (**C**). DNA fragmentation was assayed using an ELISA kit (**D**). Apoptotic cells were quantified using flow cytometry. Each value represents the mean ± SEM for n = 3. The symbols ''*'' and ''#'' indicate that a value significantly ($P < 0.05$) differs from the respective control and endogenous NO-treated groups, respectively.

human chondrocytes as was seen with SNP. Suppression of endogenous NO synthesis by NMMA significantly lowered NO-induced alterations in cellular Cyt *c* levels, caspase-3 activation, DNA fragmentation, and cell apoptosis. During inflammation, human chondrocytes can be damaged by overproduction of NO [Fermor et al., 2005]. A previous study reported that NO selectively suppresses mitochondrial complex IV enzyme activity and induces chondrocyte injuries [Maneiro et al., 2005]. This study further demonstrated that both exogenous and endogenous NO can induce apoptosis of human chondrocytes via a mitochondria-dependent mechanism. However, NO combined with ROS possibly induces necrosis of chondrocytes [Kuhn et al., 2004]. Thus, our present results do not exclude the possibility of NO-induced necrosis of human chondrocytes.

NO can cause cartilage degeneration possibly via induction of chondrocyte apoptosis. Administration of SNP, GSNO, or a combination of LPS and IFN-γ increased the proportions of human chondrocytes arrested at the sub-G1 phase. The appearance of a hypodiploid sub-G1 peak indicates that cells are undergoing apoptosis [Lee et al., 2005]. Our previous studies showed that NO can drive osteoblasts prepared from neonatal rat calvaria to undergo apoptosis [Chen et al., 2002, 2005a; Chang et al., 2006]. In this study, analysis of DNA fragmentation demonstrated that both exogenous and endogenous NO induced fragmentation of genomic DNA. A previous study reported that NO is involved in the pathophysiological regulation of infected chondrocytes [Lee et al., 2005]. Therefore, this study further demonstrated that NO induces the death of human chondrocytes via an apoptotic mechanism. In 24-h treated human chondrocytes, exogenous NO induced a greater proportion of cells to undergo apoptosis, but other factors, for example, cyanide ion, may have also contributed to the cell damage recorded. Previous studies showed that the degree of chondrocyte damage is directly correlated to the severity of human osteoarthritic cartilage [Aigner and Kim, 2002; Murray et al., 2004]. Therefore, apoptosis of human chondrocytes induced by exogenous or endogenous NO may be one of the critical factors leading to

joint degeneration when articular cartilage tissues experience traumatic or inflammatory conditions.

Mitochondrial dysfunction contributes to the NO-induced apoptosis of human chondrocytes. SNP inhibited the mitochondrial membrane potential in concentration- and time-dependent manners. By comparison, treatment with a combination of LPS and IFN-γ for 1 h did not affect the membrane potential of mitochondria because of the absence of induction of endogenous NO at this treatment time interval. However, in parallel with the increase in cellular NO levels, the mitochondrial membrane potential significantly decreased after administration of LPS and IFN-γ for 6, 12, and 24 h. Mitochondria are important ATP-synthesizing organelles. Disruption of the mitochondrial membrane potential can lead to depolarization of the mitochondrial membrane, blocking of the respiratory chain reaction, suppression of ATP synthesis, and ultimately mitochondrial dysfunction [Yu et al., 2002; Papucci et al., 2003]. Consequently, mitochondrial dysfunction usually triggers cells to undergo apoptosis [Blom et al., 2003]. Maneiro et al. [2005] showed that NO can suppress the respiratory chain reaction, and induces chondrocyte damage. Therefore, NO-induced apoptotic insults to human chondrocytes are involved in mitochondrial dysfunction due to suppression of the mitochondrial membrane potential.

Intracellular ROS mediate NO-induced apoptosis of human chondrocytes. Administration of exogenous and endogenous NO concentration- and time-dependently increased intracellular ROS levels in human chondrocytes. Depolarization of the mitochondrial membrane leads to the release of ROS from mitochondria to the cytoplasm, and mitochondrial ROS can mediate cell death signaling [Fleury et al., 2002]. Li et al. [2003] showed that inhibiting NADH dehydrogenase activity by rotenone enhances mitochondrial ROS and induces cell apoptosis. Thus, ROS are considered to be one of the mitochondrial apoptotic factors. Increases in intracellular ROS levels augment cellular oxidative stress. ROS can directly damage cellular biomolecules, for example, DNA and proteins, and induce cell apoptosis [Lu et al., 2005]. In addition, intracellular ROS have been reported to induce cell apoptosis through regulation of apoptosis-related molecules [Takahashi et al., 2004]. Results of this study show that increases in intracellular ROS simultaneously lead to apoptosis by human chondrocytes. Therefore, enhancement of intracellular ROS is one of the critical factors participating in cell apoptosis induced by exogenous or endogenous NO.

NO stimulates Cyt *c* release from mitochondria, and sequentially triggers apoptotic events. After administration of either SNP, GSNO, or a combination of LPS and IFN-γ, the release of Cyt *c* from mitochondria to the cytoplasm was significantly augmented. Mitochondrial depolarization has been reported to enlarge membrane pore sizes and cause the release of Cyt *c* to the cytoplasm [Hortelano et al., 1999]. Thus, the NO-induced suppression of the mitochondrial membrane potential in human chondrocytes is one of the crucial reasons explaining the increase in Cyt *c* release after exposure to NO. The present data cannot explain why endogenous NO has the potential to induce the release of Cyt *c* earlier than exogenous NO. The release of mitochondrial Cyt *c* has been shown to be positively related to activation of cellular proteases [Goyal, 2001]. A recent study demonstrated that Cyt *c* can mediate electron transfer between itself and the p66Shc protein to produce ROS, triggering mitochondria-dependent apoptosis [Giorgio et al., 2005]. Therefore, when exposed to exogenous and endogenous NO, Cyt *c* release is a key effector inducing cellular ROS, protease activation, and cell apoptosis.

Activation of caspase-3 plays a critical role in NO-induced apoptotic damage. Exposure of human chondrocytes to either SNP, GSNO, or a combination of LPS and IFN-γ time-dependently increased caspase-3 activity. Caspase-3 is a key protease in the processing of cells undergoing apoptosis [Goyal, 2001]. Release of Cyt *c* from mitochondria to the cytoplasm can promote the digestion of cytosolic upstream procaspase into activated subunits [Hortelano et al., 1999]. After sequential digestion events, caspase-3 is activated and contributes to the activation of DNase and DNA fragment factors, which induce DNA injuries [Liu et al., 1998]. In this study, we show that exogenous or endogenous NO time-dependently induced DNA fragmentation of human chondrocytes. Suppression of caspase-3 activation by its specific inhibitor, Z-DEVD-FMK, significantly decreased endogenous and exogenous NO-induced DNA fragmentation and cell apoptosis. Therefore, NO-involved caspase-3 activation

following the release of mitochondrial Cyt *c* participates in the signal-transducing apoptotic events in human chondrocytes.

In summary, this study shows that NO from both exogenous and endogenous sources can cause the death of human chondrocytes via an apoptotic mechanism. Sequential events occurring after exposure to NO include suppression of the mitochondrial membrane potential, increases in intracellular ROS levels, augmentation of Cyt *c* release, activation of caspase-3, and induction of DNA fragmentation. In conclusion, endogenous and exogenous NO can induce apoptotic insults to human chondrocytes via a mitochondrion-dependent mechanism. In a future study, we will provide more evidence to further show the effects of exogenous and endogenous NO on apoptotic insults to human chondrocytes and their possible molecular mechanisms.

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