Apoptotic Insults to Human Chondrocytes Induced by Sodium Nitroprusside Are Involved In Sequential Events, Including Cytoskeletal Remodeling, Phosphorylation of Mitogen-Activated Protein Kinase Kinase Kinase-1/c-Jun N-Terminal Kinase, and Bax-Mitochondria-Mediated Caspase Activation

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ABSTRACT: Nitric oxide (NO) can regulate chondrocyte activities. This study was aimed to evaluate the molecular mechanisms of NO donor sodium nitroprusside (SNP)-induced insults to human chondrocytes. Exposure of human chondrocytes to SNP increased cellular NO levels but decreased cell viability in concentration- and time-dependent manners. SNP time dependently induced DNA fragmentation and cell apoptosis. Treatment with 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl 3-oxide, an NO scavenger, significantly lowered SNP-induced cell injuries. Administration of SNP interrupted F-actin and microtubule cytoskeletons and stimulated phosphorylation of mitogen-activated protein kinase kinase kinase-1 (MEKK1) and c-Jun N-terminal kinase (JNK). Similar to SNP, cytochalasin D, an inhibitor of F-actin formation, disturbed F-actin polymerization and increased MEKK1 and JNK activations. Overexpression of a dominant negative mutant of MEKK1 (dnMEK1) in human chondrocytes significantly ameliorated SNP-induced cell apoptosis. Exposure to SNP promoted Bax translocation from the cytoplasm to mitochondria, but application of dnMEKK1 lowered the translocation. SNP time dependently decreased the mitochondrial membrane potential, complex I NADH dehydrogenase activity, and cellular ATP levels, but increased the release of cytochrome *c* from mitochondria to the cytoplasm. Activities of caspase-9, -3, and -6 were sequentially increased by SNP administration. This study shows that SNP can induce apoptosis of human chondrocytes through sequential events, including cytoskeletal remodeling, activation of MEKK1/JNK, Bax translocation, mitochondrial dysfunction, cytochrome c release, caspase activation, and DNA fragmentation. © 2008 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: human chondrocytes; nitric oxide; cytoskeletal remodeling; MEKK1/JNK; Bax translocation; mitochondria-dependent apoptotic mechanism

Chondrocytes are one of the crucial components for constructing cartilage tissues. A variety of systemic and local factors contribute to regulation of chondrocyte activities.[1] Nitric oxide (NO) can modulate chondrocyte metabolism and cartilage remodeling.[2] NO has biphasic effects on chondrocyte activities.[2,3] In untreated chondrocytes, NO can be constitutively produced and plays a critical role in adjusting cell proliferation and differentiation.[4,5] However, overproduction of NO has been reported to induce chondrocyte dysfunction or even death.[6,7] Apoptosis, energy-dependent cell death, plays an important role in physiological and pathophysiological regulation of tissue homeostasis and cell activities.[8] During development, apoptosis of chondrocytes participates in the morphogenetic, histogenetic, and phylogenetic processes of cartilage tissue.[5]

There are many apoptotic factors involved in programmed cell death.[8] Cytoskeletons are crucial organelles for maintenance of cellular morphologies, polarity, and movement.[9] Recent studies have revealed that changes in the dynamics of cytoskeletal remodeling can induce cell apoptosis.[10,11] Gourlay et al.[12] showed that disturbances of the F-actin cytoskeleton resulted in mitochondrial dysfunction, release of reactive oxygen species, and cell death. Amyloid β-peptide can sequentially stimulate the perturbation of the microtubule cytoskeleton, proteolysis of microtubule-associated proteins, and consequent induction of neuronal apoptosis.[13] Mitogen-activated protein kinase kinase kinase-1 (MEKK1), an upstream regulator of mitogen-activated protein kinases (MAPKs) that comprise c-Jun N-terminal kinase (JNK), orchestrates the effects of many extracellular stimuli on cells.[14] MEKK1 has been reported to transduce actin signals in keratinocytes to induce fiber formation and migration.[15]

Mitochondria, energy-producing organelles, can regulate the process of cell apoptosis.[16,17] Our previous studies showed that NO induces osteoblast apoptosis via a mitochondria-dependent mechanism.[18–20] In chondrocyte-like ATDC5 cells, energy depletion induced by mitochondrial dysfunction has been shown to mediate interleukin-1β-triggered cell apoptosis.[21] Bax and cytochrome *c* are mitochondria-related apoptotic factors.[16,22] Increases in the synthesis or translocation of Bax, a proapoptotic protein, can trigger depolarization of the mitochondrial membrane potential, enhancing the release of cytochrome *c*, and ultimately leading to cell apoptosis.[23] Phosphorylation of MAPKs by MEKK1 has been reported to activate the Bax–caspase protease pathway and plays a pivotal role in high glucose-induced apoptosis of human endothelial cells.[24] However, the roles

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of the cytoskeleton and MEKK1/JNK in NO-induced insults to chondrocytes need to be evaluated.

During inflammation, reactive oxygen species can be overproduced by chondrocytes themselves and surrounding cells, and induces cell injuries.[3,5–7] NO radical is one of the important reactive oxygen species. In osteoblasts, NO from endogenous or exogenous sources has been shown to induce cell apoptosis via a Bax–mitochondria–caspase protease pathway.[18–20] Investigating NO-induced chondrocyte insults is crucial to the clinical treatment of cartilage dysfunction. However, the detailed molecular mechanisms of NO-induced chondrocyte injuries still need to be elucidated. Therefore, this study was designed to evaluate the signal-transducing mechanisms of NO-induced chondrocyte injuries from the viewpoints of cytoskeletal remodeling, phosphorylation of MEKK1/JNK, Bax translocation, mitochondrial dysfunction, and cytochrome *c*-mediated caspase activation.

MATERIALS AND METHODS

Cell Culture, Drug Treatment, and Viability Assay

Human chondrocytes were purchased from Cell Applications (San Diego, CA). The cell line was derived from normal human articular cartilage and can be cultured and propagated through at least 10 population doublings. The cells were seeded in chondrocyte growth medium (Cell Applications), which is fully supplemented for culturing and propagating cells. Human chondrocytes were cultured in 75-cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2. Our preliminary data showed that this cell line can constitutively express collagen type II mRNA and protein (data not shown). Sodium nitroprusside (SNP), an NO donor, was purchased from Sigma (St. Louis, MO) and freshly dissolved in phosphate-based saline (PBS) buffer (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) and protected from light. To confirm the roles of NO in cell insults, human chondrocytes were treated with a combination of SNP and 100 μM 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), an NO scavenger, for 24 h.

Levels of cellular NO in human chondrocytes were determined according to the technical bulletin of the Bioxytech NO assay kit (OXIS International, Portland, OR) as described previously.[18] In this kit, nitrate reductase is provided to reduce nitrate to nitrite so total nitrite in the culture medium was detected. A trypan blue exclusion method was carried out to determine the cytotoxicity of SNP to human chondrocytes. The cells on a haemacytometer were counted and analyzed.

Quantification of DNA Fragmentation and Apoptotic Cells

DNA fragmentation in human chondrocytes was quantified to evaluate if SNP damages nuclear DNA as described previously.[25] 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). Apoptotic cells were determined by detecting cells which were arrested at the sub-G1 stage according to a previously described method.[19]

Confocal Microscopic Analysis of the F-Actin and Microtubule Cytoskeletons

The F-actin and microtubule cytoskeletons in human chondrocytes were visualized using confocal microscopy. Briefly, after drug treatment, the cells were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton X-100. For imaging analysis of F-actin filaments, cells were stained with 0.5 μg/mL phalloidin-FITC (Molecular Probes, Eugene, OR). For imaging analysis of microtubule cytoskeleton, human chondrocytes were immunodetected using a mouse monoclonal antibody labeled with FITC against mouse α-tubulin (Molecular Probes). A confocal laser scanning microscope (Model FV500, Olympus, Tokyo, Japan) was utilized for sample observation. Images were acquired using the FLUOVIEW software (Olympus). Control cells received PBS buffer only, and the buffer did not affect the cytoskeletons.

Immunodetection of Phosphorylated and Nonphosphorylated MEKK1, JNK, and Cytochrome *c*

After drug treatment, cell lysates were prepared in 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 protein assay kit (Pierce, Rockford, IL). Cytosolic proteins (50 μg per well) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Immunodetection of phosphorylated MEKK1 was carried out using a rabbit polyclonal antibody with a synthetic phosphopeptide corresponding to residues surrounding Thr286 of the human MEKK1 protein (Cell Signaling, Danvers, MA). Cellular MEKK1 was immunodetected using a mouse monoclonal antibody against human MEKK1 (Cell Signaling) as the internal standard. Phosphorylated JNK was immunodetected using a rabbit polyclonal antibody with a synthetic phosphopeptide corresponding to residues Thr183/Tyr185 of human JNK (Cell Signaling). JNK was detected using a mouse monoclonal antibody against human JNK (Cell Signaling) as the internal standard. Cytochrome *c* protein was immunodetected using a mouse monoclonal antibody against rat cytochrome *c* protein (Transduction Laboratories, Lexington, KY). β-Actin was immunodetected by a mouse monoclonal antibody against mouse β-actin (Sigma) as an internal control. Intensities of the immunoreactive protein bands were determined using a digital imaging system (UVtec, Cambridge, UK).

Establishment of Human Chondrocyte/dnMEKK1 Clones

Glucocorticoid-inducible pSRα-MEKK1 (K432 M) vectors, a gift from Dr. Michael Karin of the Department of Pharmacology, School of Medicine, University of California (San Diego, La Jolla, CA), were transfected using the Lipofectin Reagent (Invitrogen, Carlsbad, CA) into human chondrocytes. To avoid problems with clonal variations, the transfected cells were selected using hygromycin for 4 weeks, and all of the clones were pooled as described previously.[26] Control cells were transfected with empty vectors.

Confocal Microscopic Analysis of Bax Translocation

After drug treatment, human chondrocytes were fixed, rehydrated, and reacted with 0.2% Triton X-100. Bax was immunodetected using a mouse monoclonal antibody against human Bax (Santa Cruz Biotechnology, Santa Cruz, CA) as described previousely.[27] Cells were sequentially reacted with the biotin SP-conjugated second antibody and with the Cy3-streptavidin-conjugated third antibody (Jackson ImmunoResearch, West Grove, PA). Mitochondria of human chondrocytes were stained with 3,3′-dihexyloxacarbocyanine ($DiOC_6$), a positively charged dye (Molecular Probes).[28] A confocal laser scanning microscope (Olympus) was utilized for

sample observation. Images were acquired using the FLUOVIEW software (Olympus).

Assays of Mitochondrial Membrane Potential, NADH Dehydrogenase Activity, and Cellular ATP Levels

The membrane potential of mitochondria in human chondrocytes was determined according to a previously described method.[13] Briefly, after drug administration, human chondrocytes were harvested and incubated with $DiOC_6$ at 37°C for 30 min in a humidified atmosphere of 5% CO_2. After washing and centrifugation, cell pellets were suspended in PBS buffer. Intracellular fluorescent intensities were analyzed using a flow cytometer. Mitochondrial NADH dehydrogenase activity was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay following the method of Wu et al.[19] Cellular ATP levels were determined by a bioluminescence assay as described previously.[29]

Fluorogenic Substrate Assay for Caspase Activities

Activities of caspase-3, -6, and -9 were determined using fluorogenic assay kits (R&D Systems, Minneapolis, MN). After drug treatment, human chondrocytes were lysed, and the cell extracts (25 μg total protein) were incubated with 50 μM specific fluorogenic peptide substrates, DEVD, VEID, and LEHD for caspase-3, -6, and -9, respectively. Intensities of fluorescent products were measured by the LS 55 spectrometer of PerkinElmer Instruments (Shelton, CT).

Statistical Analyses

The statistical difference between 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

Exposure of human chondrocytes to 0.1, 0.5, and 1 mM SNP for 24 h significantly increased cellular NO levels by 38%, and 3.1- and 6.8-fold, respectively. When exposed to 1 mM SNP for 1, 6, 12, and 24 h, the levels of cellular NO were augmented by 27 and 86%, and 6.7- and 8.8-fold, respectively (data not shown). In parallel with the increases of cellular NO levels, exposure to 0.5 and 1 mM SNP for 24 h decreased cell viability by 38 and 74%, respectively (Fig. 1A, top panel). After treatment with 1 mM SNP for 6, 12, and 24 h, cell viability decreased by 28, 50, and 69%, respectively (Fig. 1A, bottom panel). Administration of 1 mM SNP for 6, 12, and 24 h significantly increased DNA fragmentation by

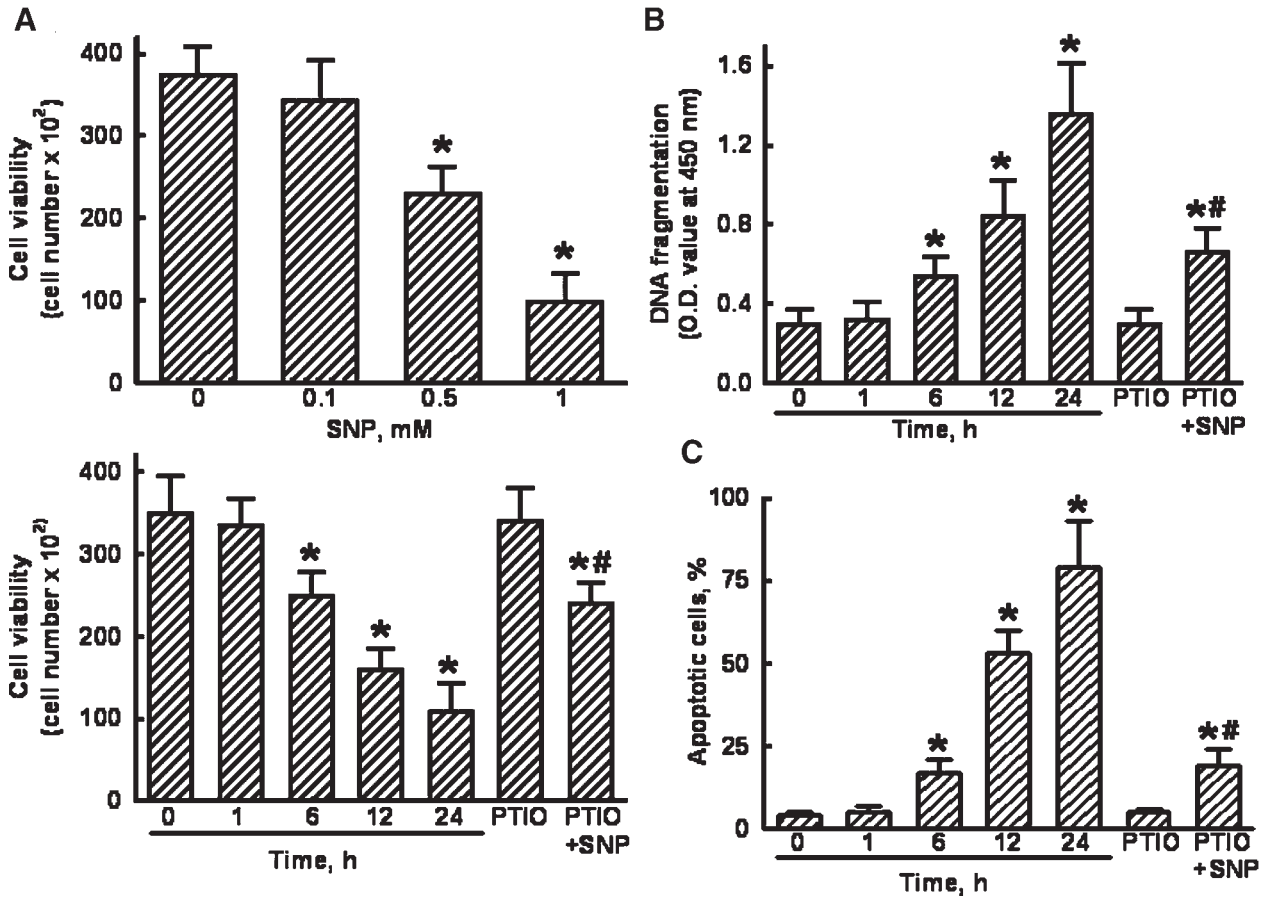

Figure 1. Cytotoxic effects of sodium nitroprusside (SNP) on viability. DNA fragmentation, and apoptosis of human chondrocytes. Human chondrocytes were exposed to 0.1, 0.5, and 1 nM SNP for 24 h or to 1 nM SNP for 1, 6, 12, and 24 h. To confirm the roles of nitric oxide in cell insults, human chondrocytes were treated with a combintaiton of SNP and 2-phenyl-4,4,5,5,tetramethyl-imidazoline-1-oxyl 3-oxide (PTIO) a nitric oxide scavenger, for 24 h. Cell viability was determined using the trypan blue exclusion method (A). DNA fragmentation was quantified using an enzyme-linked immunosorbent assay (B). The proporation of apoptotic cells was detected using flow cytometry (C). The passage number of human chondrocytes used for these assays was less than 10. Each value represents the mean ± SEM, n = 8. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and SNP-treated groups, respectively.

80%, and 2.8- and 4.5-fold, respectively (Fig. 1B). When exposed for 6, 12, and 24 h, SNP caused significant 17, 53, and 78% increases in apoptotic cells (Fig. 1C). Treatment of human chondrocytes along with PTIO did not cause cell damage (Fig 1A–C). Meanwhile, exposure to PTIO respectively alleviated SNP-induced alterations in cell viability, DNA fragmentation, and apoptosis by 54, 51, and 59%.

F-Actin filaments and the microtubule structure were analyzed to determine the effects of SNP on cytoskeletons of human chondrocytes (Fig. 2). In untreated human chondrocytes, the long-form and regular F-actin filaments were observed (Fig. 2A). After administration of SNP for 1 h, the polymerization of F-actin filaments was interrupted. After exposure for 6, 12, and 24 h, SNP not only shortened F-actin filaments but also induced cell shrinkage. Cytochalasin D, an inhibitor of F-actin polymerization, caused interruption of F-actin filaments (Fig. 2A). In untreated human chondrocytes, the microtubule cytoskeleton was uniformly distributed (Fig. 2B). After treatment with SNP for 1 h, the structure of the microtubule cytoskeleton was disturbed. When the administered time intervals reached 6, 12, and 24 h, SNP induced disruption of microtubule structure and cell shrinkage. Colchicines was used here as the positive reagent for triggering interruption of microtubule remodeling (Fig. 2B).

The roles of MEKK1 and JNK in NO-induced apoptosis were determined using immunoblotting and a dominant negative analysis (Fig. 3). After administration for 1 h, SNP significantly increased the amounts of phosphorylated MEKK1 in human chondrocytes, and the enhanced effect lasted for 2 h (Fig. 3A, top panel, lanes 2 and 3). In parallel with cytoskeletal interruption, cytochalasin D stimulated phosphorylation of MEKK1 (lane 4). Nonphosphorylated MEKK1 was immunodetected as the internal control (Fig. 3A, bottom panel). These protein bands were quantified and analyzed, and

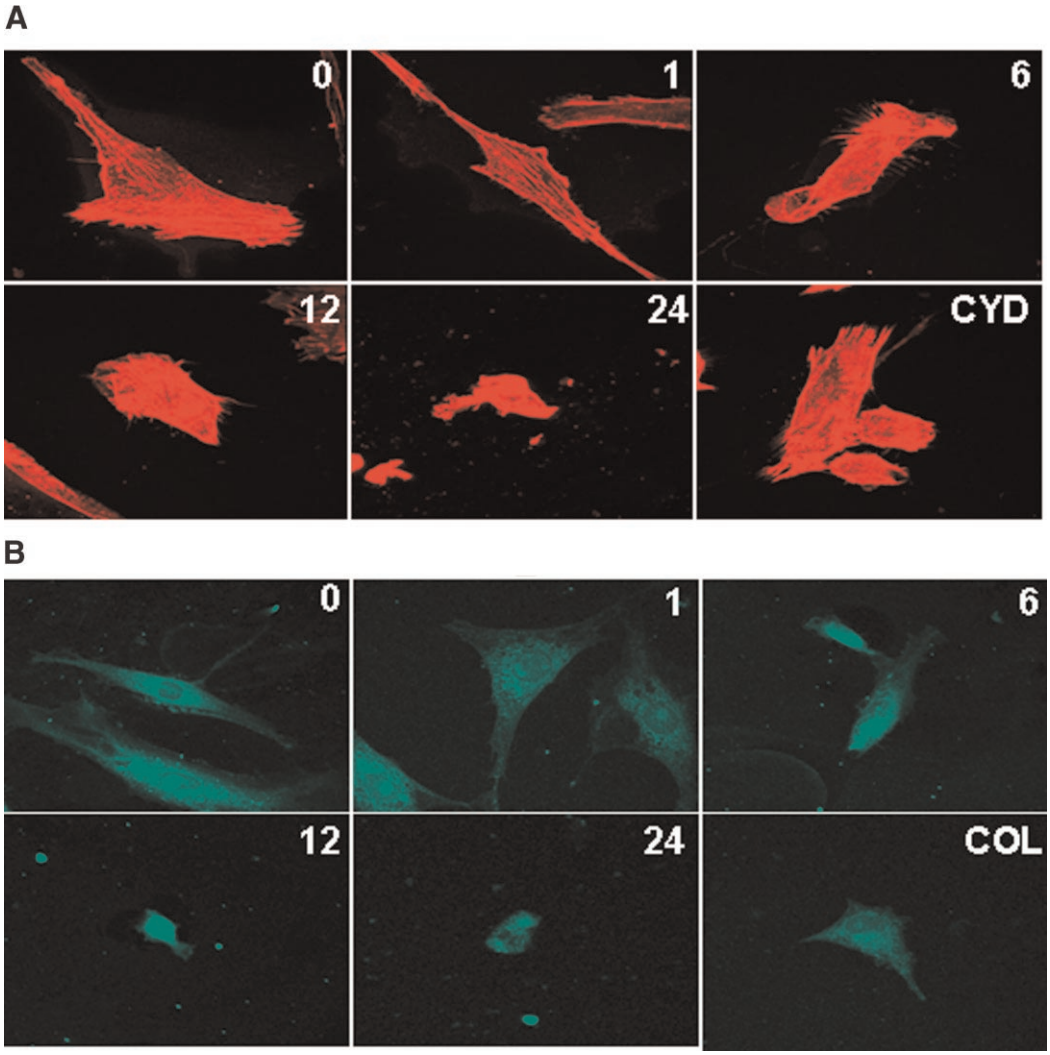

Figure 2. Time-dependent effects of sodium nitroprusside (SNP) on F-actin and microtubule cytoskeletons. Human chondrocytes were exposed to 1 mM SNP for 1, 6, 12, and 24 h. The F-actin filaments in human chondrocytes were stained with phalloidin-FITC and visualized using confocal microscopy (A). The microtubule cytoskeleton in human chondrocytes was immunodetected using a mouse monoclonal antibody labeled with FITC against mouse α-tubulin and observed using confocal microscopy (B). Cytochalasin D (CYD) and colchicines (COL) were applied to the cells for 1 h as the positive control for inhibiting F-actin and microtubule cytoskeletons, respectively. The passage number of human chondrocytes used for these assays was less than 10.

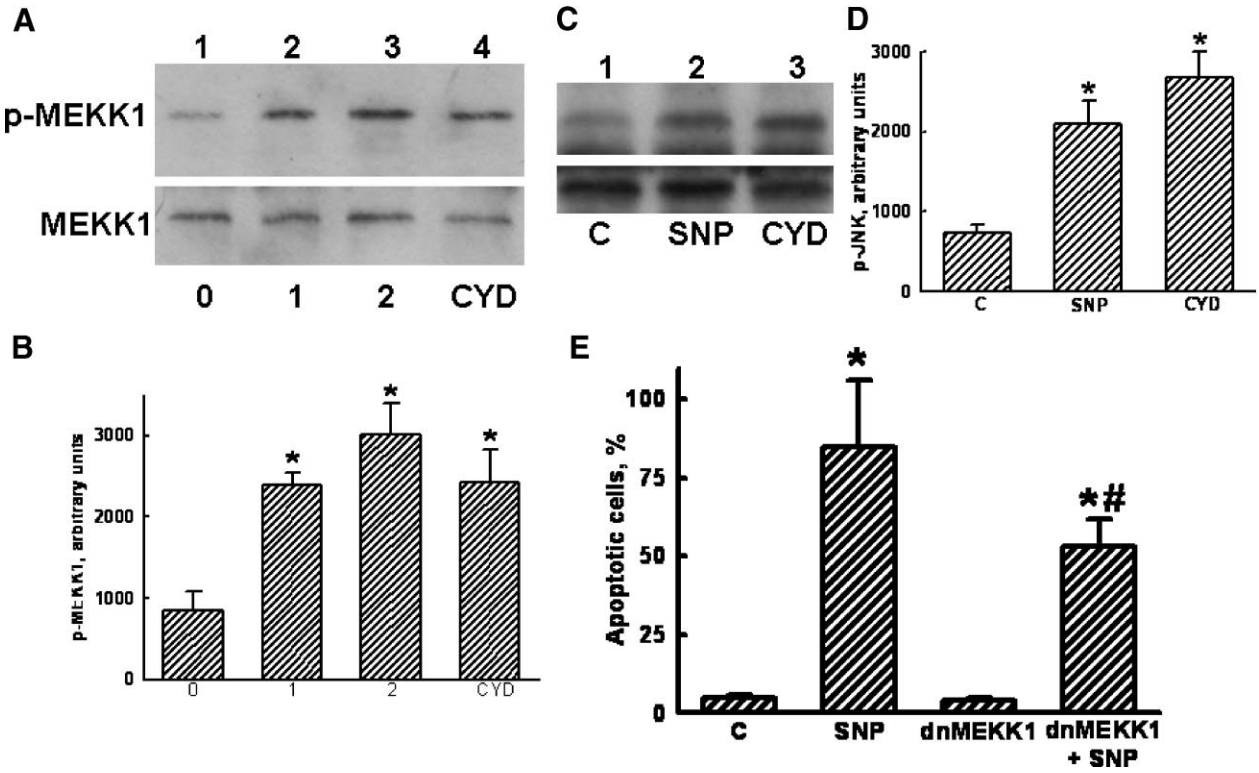

Figure 3. Effects of sodium nitroprusside (SNP) on phosphorylation of MEKK1 and JNK. Human chondrocytes were exposed to 1 mM SNP for 0, 1, and 2 h (lanes 1–3) or to cytochalasin D (CYD) for 1 h (lane 4). Phosphorylated MEKK1 was immunodetected (A, top panel). MEKK1 was detected as the internal standard (A, bottom panel). The cells were treated with SNP and CYD for 2 h, and phosphorylated JNK was determined (C, top panel, lanes 2 and 3). JNK was quantified as the internal standard (C, bottom panel). Intensities of these immunorelated protein bands were quantified by a digital system (B and D). The role of MEKK1 in NO-induced apoptotic insults to human chondrocytes was evaluated by subjecting a dominant negative mutant of MEKK1 (dnMEKK1) in the cells, and apoptotic analysis was carried out (E). The passage number of human chondrocytes used for these assays was less than 10. Each value represents the mean ± SEM, n = 4. The symbols * and # indicate that a value significantly ($p < 0.05$) differs from the respective control and SNP-treated groups, respectively. CYD, cytochalasin D.

are shown in Figure 3B. Administration of human chondrocytes with SNP for 1 and 2 h significantly augmented the levels of phosphorylated MEKK1 by 2.8- and 3.5-fold, respectively. Exposure to cytochalasin D for 1 h caused a significant 2.8-fold increase in the phosphorylated MEKK1 level (Fig. 3B). Sequentially, exposure to SNP and cytochalasin D significantly increased the levels of phosphorylated JNK by 2.6- and 2.9-fold, respectively (Fig. 3C and D).

To further evaluate the role of MEKK1 in NO-induced apoptotic insults to human chondrocytes, dnMEKK1 was administered to cells, and an apoptotic analysis was carried out (Fig. 3E). Administration of SNP significantly induced 85% of human chondrocytes to undergo apoptosis. Subjection of dnMEKK1 to human chondrocytes did not affect cell apoptosis. Overexpression of dnMEKK1 significantly lowered SNP-induced apoptosis of human chondrocytes by 42% (Fig. 3E).

Translocation of Bax from the cytoplasm to mitochondria was visualized to determine the effects of NO on activation of this proapoptotic protein (Fig. 4). Administration of SNP obviously enhanced Bax protein (Fig. 4). In parallel with the increases in the levels of this proapoptotic protein, exposure to SNP increased the translocation of Bax from the cytoplasm to mitochondria. Overexpression of dnMEKK1 in human chondrocytes suppressed the SNP-induced Bax translocation from the cytoplasm to mitochondria (Fig. 4).

To determine the effects of NO on mitochondrial function, the mitochondrial membrane potential, complex I NADH dehydrogenase activity, cellular ATP levels, and cytochrome *c* release were quantified (Fig. 5). Exposure of human chondrocytes to SNP for 1, 6, 12, and 24 h decreased the mitochondrial membrane potential by 15, 28, 38, and 50%, respectively (Fig. 5A). Activities of mitochondrial complex I NADH dehydrogenase were suppressed by 31, 40, 47, and 55% following administration of SNP for 1, 6, 12, and 24 h, respectively (Fig. 5B). Treatment with SNP for 1, 6, 12, and 24 h significantly decreased cellular ATP levels by 33, 42, 52, and 67%, respectively (Fig. 5C). The levels of cellular cytochrome c were augmented by 2-, 3.6-, 3-, and 2.7-fold after exposure to SNP for 1, 6, 12, and 24 h, respectively (Fig. 5D).

Activities of caspase-9, -3, and -6 were assayed to determine the signal-transducing mechanism of NO-induced cell apoptosis (Fig. 6). Exposure to 1 mM SNP for 6, 12, and 24 h significantly increased caspase-9 activities by 2-, 2.3-, and 2.1-fold, respectively (Fig. 6A). After SNP administration for 6, 12, and 24 h, caspase-6 activity was augmented by 57%, twofold, and 75%, respectively (Fig. 6B). The activities of caspase-3 were respectively enhanced by 76%, and 2.6- and 2.1-fold

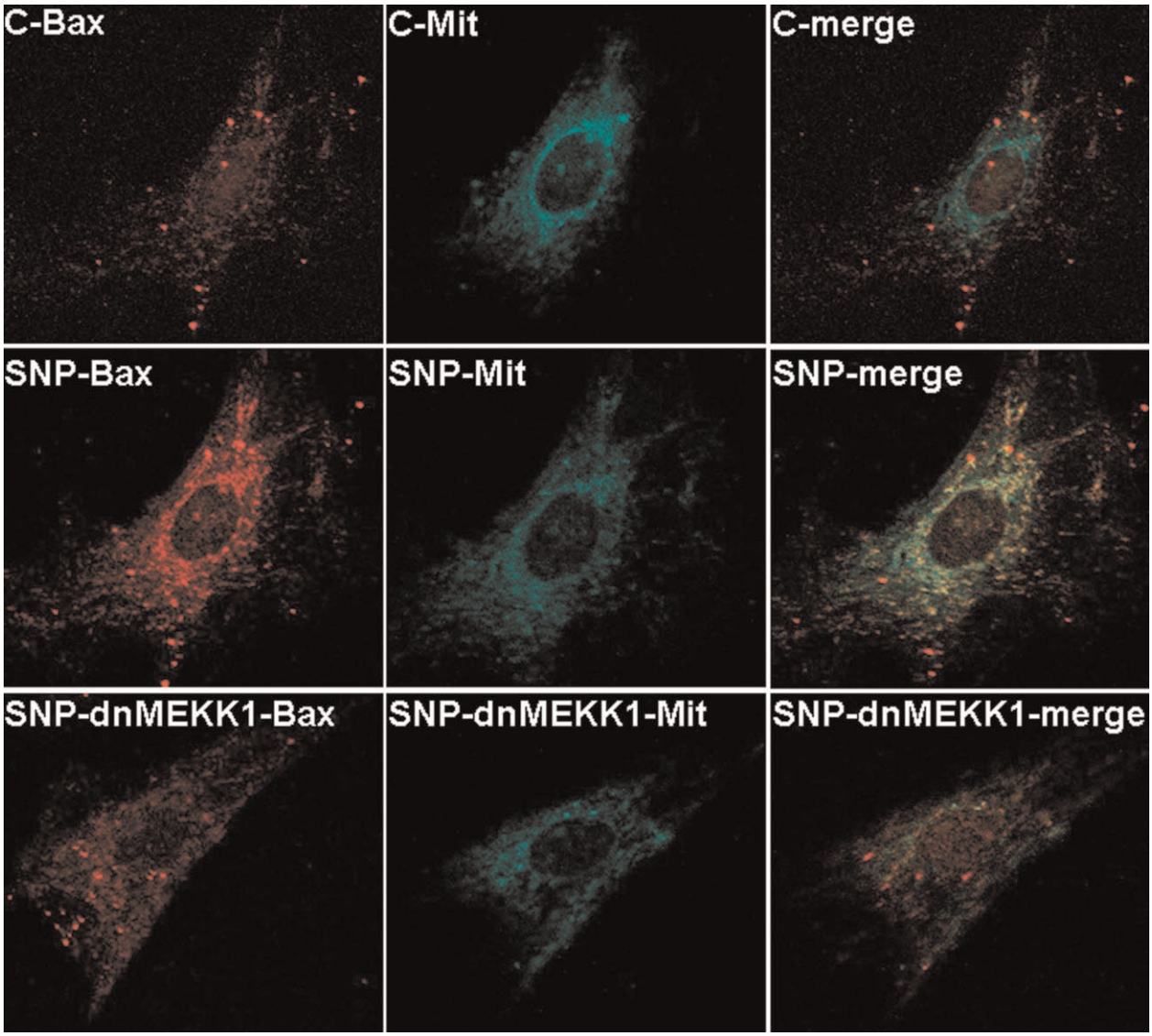

Figure 4. Effects of sodium nitroprusside (SNP) and a dominant negative mutant of MEKK1 (dnMEKK1) on Bax translocation. Human chondrocytes were exposed to SNP or dnMEKK1. The distribution of Bax protein was immunodetected using an antibody with Cy3-conjugated streptavidin. The mitochondria of human chondrocytes were stained with $DiOC_6$, a positively charged dye. The fluorescent images were visualized using a confocal laser scanning microscope. C, control; Mit, mitochondria. The passage number of human chondrocytes used for these assays was less than 10.

following SNP administration for 6, 12, and 24 h, respectively (Fig. 6C).

DISCUSSION

The present data from analyses of cell viability, DNA fragmentation, cell apoptosis, and NO scavenging reveal that NO decomposed from SNP caused insults to human chondrocytes via an apoptotic pathway. However, PTIO could not completely alleviate SNP-induced chondrocyte insults. del Carlo and Loeser[3] reported that NO combined with just other reactive oxygen species could cause chondrocyte death. Thus, NO decomposed from SNP possibly reacted with superoxide to form peroxynitrite and simultaneously induced chondrocyte apoptosis. The concentrations of SNP used in this study were high. Our previous studies showed that pretreatment with low concentrations of SNP (<0.3 mM) for 24 h did not cause cell injuries but could protect osteoblasts from high concentrations of SNP (>1 mM)-induced apoptotic insults.[20,28] A previous study demonstrated that increased oxidative stress caused dysfunction of the glutathione antioxidant system and decreased chondrocyte survival.[30] Kim et al.[31] showed that NO induced chondrocyte apoptosis via p38 kinase-mediated inhibition of protein kinase C zeta. The present study further provides in vitro data to show that NO decomposed from SNP induced apoptosis of a human chondrocyte cell line through sequential events, including cytoskeletal remodeling, phosphorylation of MEKK1/JNK, and activation of the Bax–mitochondria–caspase protease pathway.

Previous studies reported that an imbalance of cytoskeletal remodeling leads to cell dysfunction or even death.[10,32] Our present data reveal that SNP disturbed

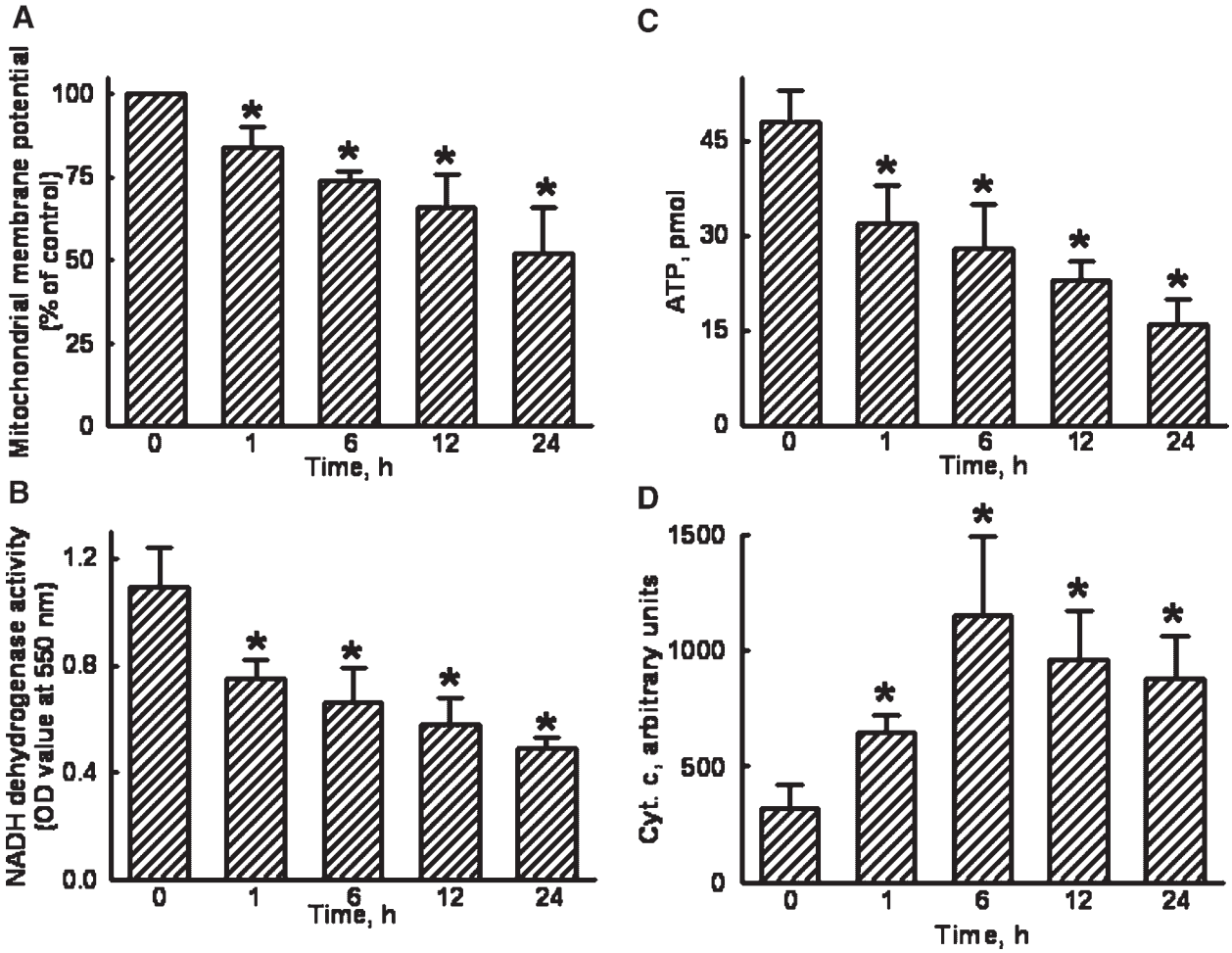

Figure 5. Effects of sodium nitroprusside (SNP) on the mitochondrial membrane potential, NADH dehydrogenase activity, cellular ATP levels, and release of cytochrome *c* (Cyt. C). Human chondrocytes were exposed to 1 mM SNP for 1, 6, 12, and 24 h. The mitochondrial membrane potential was stained with $DiOC_6$ and quantified using flow cytometry (A). The activity of mitochondrial complex I NADH dehydrogenase was assayed using a colorimetric method (B). Levels of cellular ATP were quantified using a bioluminescence assay (C). The amounts of cytochrome *c* were immunodetected using a monoclonal antibody (D). The passage number of human chondrocytes used for these assays was less than 10. Each value represents the mean ± SEM, n = 6. *Values significantly differ from the respective control, $p < 0.05$.

F-actin and microtubule cytoskeletons but did not affect cell viability in 1 h-treated human chondrocytes. After administration of SNP for 6 h, the interruption of the F-actin and microtubule cytoskeletons became much worse, and the viability of human chondrocytes decreased. Thus, the perturbation of cytoskeletal remodeling may be an upstream event in SNP-induced insults. The results from detection of kinase phosphorylation and a dominant negative assay further showed that MEKK1 can mediate the signal from F-actin cytoskeletons to induce chondrocyte apoptosis. Therefore, cytoskeletal remodeling and MEKK1 activation play initiating roles in SNP-induced chondrocyte apoptosis.

MEKK1 is reported to mediate extracellular stimuli via sequential phosphorylation of downstream protein kinases such as MAPKs to regulate physiological and pathophysiological conditions of cells.[14] SNP and cytochalasin D can activate JNK, one of MAPKs. Harnois et al.[33] reported that MAPK activation leads to an increases in the levels of cellular Bax protein and contributes to apoptosis of human intestinal epithelial crypts. This study further demonstrates that the SNP-caused enhancement in Bax translocation is related to activations of MEKK1 and JNK. The Bax protein is translocated to mitochondria from the cytoplasm and then insert itself into the outer mitochondrial membrane, permeabilizing the membrane, triggering the release of mitochondria-related apoptotic factors, and inducing cell apoptosis.[24,34] SNP administration significantly decreased the mitochondrial membrane potential and increased cytochrome *c* release. Therefore, SNP can enhance cytochrome *c* release due to the depolarization of mitochondrial membranes induced by MEKK1/JNK-involved Bax translocation.

NO induces mitochondrial dysfunction and cell apoptosis. Mitochondria are important ATP-synthesizing organelles. The cellular levels of ATP in human chondrocytes were time-dependently decreased after SNP administration. Previous studies reported that disruption of the mitochondrial membrane potential leads to mitochondrial depolarization and blocks the respiratory chain reaction.[17,35] Thus, one possible mechanism involved

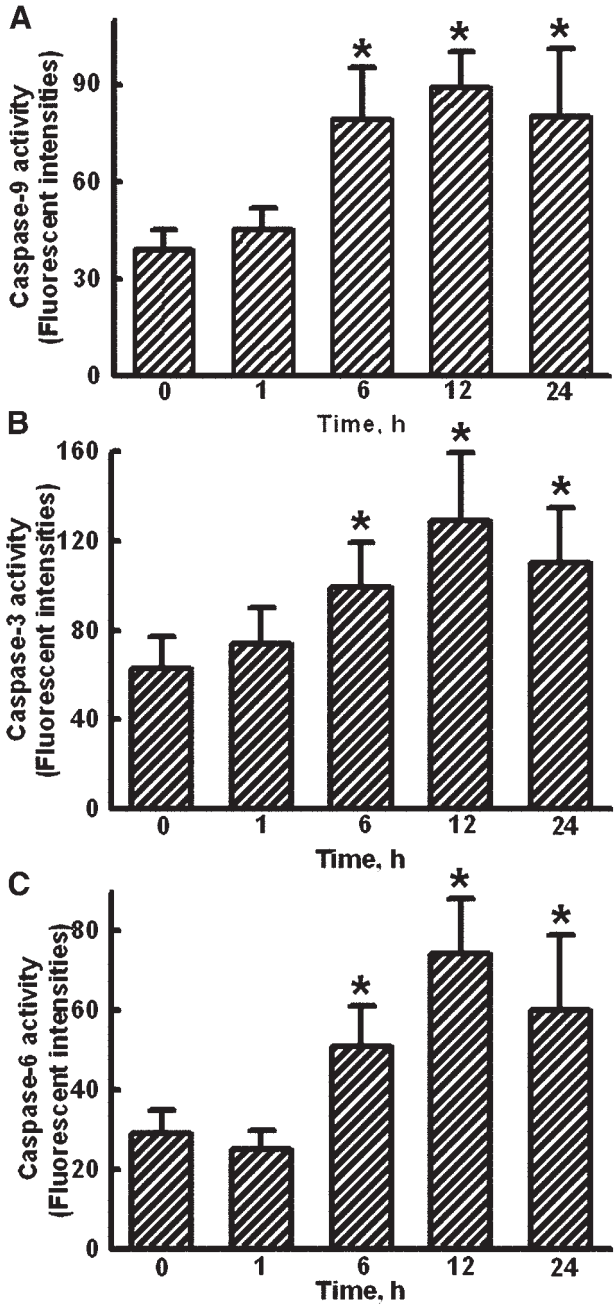

Figure 6. Time-dependent effects of sodium nitroprusside (SNP) on the activities of caspase-9, -3, and -6. Human chondrocytes were exposed to 1 mM SNP for 1, 6, 12, and 24 h. Activities of caspase-9, -3, and -6 were assayed by a fluorogenic substrate assay using DEVD, VEID, and LEHD as the respective substrates. Fluorescent intensities were analyzed by a fluorescence spectrophotometer (A–C). The passage number of human chondrocytes used for these assays was less than 10. Each value represents the mean ± SEM, n = 6. *Values significantly differ from the respective control, $p < 0.05$.

in the NO-induced depletion of ATP in human chondrocytes might be through suppression of the mitochondrial membrane potential. NADH dehydrogenase contributes to the respiratory chain reaction and ATP synthesis.[36] The suppression of NADH dehydrogenase activity might be another possible mechanism involved in the NO-induced ATP depletion in human chondrocytes. Intracellular ATP levels participate in regulation of cell apoptosis and necrosis.[23,37] Therefore, NO may decrease cellular ATP levels through suppression of the mitochondrial membrane potential and complex I enzyme activity in human chondrocytes and induces cell insults.

Cascade activation of caspase -9, -3, and -6 plays a critical role in NO-induced apoptosis of human chondrocytes. Cytochrome *c* released from mitochondria can interact with cytoplasmic apoptotic protease-activating factor-1 in forming apoptosomes and mediating caspase-9 activation.[38] Activation of caspase-9 promotes cytosolic downstream pro-caspase digestion, including caspase-3 and -6, into activated subunits.[22] Caspase-3 is a key protease in the processing of cells undergoing apoptosis.[39] After sequential digestion events, caspases-3 is activated and then cleaves cellular key proteins such as lamin and nuclear mitotic apparatus proteins to affect cell functions.[40] Caspase-3 and -6 are reported to contribute to activation of nuclear DNase, which consequently induces fragmentation of genomic DNA.[41] Therefore, the NO-induced cascade activation of caspase -9, -3, and -6 following release of mitochondrial cytochrome *c* participates in the signal-transducing apoptosis of human chondrocytes induced by SNP.

In summary, this study shows that SNP can cause the death of human chondrocytes via an apoptotic mechanism. Sequential events occur after exposure to SNP, including interruption of F-actin and microtubule cytoskeletons, MEKK1/JNK activation, Bax translocation, reduction in the mitochondrial membrane potential, mitochondrial dysfunction, release of cytochrome *c*, activation of caspase-9, -3, and -6, and consequent induction of DNA fragmentation. In conclusion, SNP can induce apoptotic insults to human chondrocyte via a cytoskeleton–MEKK1–JNK-mediated Bax–mitochondria–caspase protease pathway. Our further study using primary porcine chondrocytes as the experimental models showed that SNP could also induce cell apoptosis via a mitochondria-dependent mechanism. However, because the human chondrocyte cell line used in this study may possess certain differences from primary chondrocytes, the conclusions of the present study could be limited.

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