Original Article

SNP-induced apoptosis may be mediated with caspase inhibitor by JNK signaling pathways in rabbit articular chondrocytes

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ABSTRACT — NO plays an important role in cartilage destruction by inducing apoptosis of chondrocytes. Here we investigated the role of c-Jun N-terminal kinase (JNK) signal transduction pathways in the apoptosis induced by NO donor sodium nitroprusside (SNP) in rabbit articular chondrocytes. We used Annexin V-FITC/PI flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) assay to detect apoptosis rate. The expressions of p38, NF- κ B p65, caspase-3 and p53 genes at protein levels were measured by Western blotting assay. RT-PCR was performed to show the mRNA expression of caspase-3, and the activity of caspase-3 was also detected. To investigate the effect of JNK-specific inhibitor SP600125, chondrocytes were pretreated with SP600125 ahead of SNP treatment. Treatment with SNP accelerated apoptosis in a concentration dependent manner, while such acceleration was reduced by SP600125 pretreatment. Moreover, we found that SP600125 significantly decreased NO-induced NF- κ B, p53, caspase-3 protein expressions and caspase-3 mRNA expression, as well as intracellular caspase-3 activity ($P < 0.05$). Collectively, these data suggest that JNK plays an important role through stimulating NF- κ B, p53 and caspase-3 activation.

Key words: Sodium nitroprusside, Apoptosis, JNK, NF- κ B, p53, Caspase-3, Chondrocytes

INTRODUCTION

Chondrocytes are the only cell type found in normal mature cartilage. The balance between anabolic and catabolic reactions regulates the phenotype of chondrocytes (Caterson *et al.*, 2000). The maintenance of cartilage homeostasis is destroyed in degenerative diseases, such as osteoarthritis (OA) and rheumatoid arthritis (RA). It is characterized by deteriorative structural changes in cartilage, leading to loss of joint function (Kraan and Berg, 2000). Destruction and loss of articular cartilage is a central feature of OA and RA (Loeser, 2006). It has become increasingly clear in recent years that multiple cell types contribute to joint structure and function contribute to OA and RA.

Reactive oxygen species (ROS) and nitric oxide (NO)

have been hypothesized to contribute to the development of OA. NO is involved in the functional regulation of cartilage at multiple levels. And patients suffering from rheumatic diseases have showed the concentrations of nitrite in synovial fluid and serum was high (Feelisch, 2008). Overproduction of NO in articular chondrocytes has been reported to induce chondrocyte apoptosis. NO is produced in large amounts by chondrocytes upon proinflammatory cytokine stimulation (Clancy *et al.*, 1997) and causes apoptotic cell death (Kim *et al.*, 2002a). Inhibition of NO production protects cartilage and chondrocytes against damage (Wu *et al.*, 2007). However, the signaling pathway leading to NO-induced apoptosis of chondrocyte has not been well characterized.

The mitogen-activated protein kinase (MAPK) members have been identified as an important mediator of

signal transduction. It is activated by proinflammatory cytokines, which additionally generate NO, suggesting the role for NO in MAP kinase signaling. Among the MAPK family, 3 members have been well characterized: the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAP kinases (Gulmann *et al.*, 2009). Each MAPK is activated through the specific kinase cascade. The ERK signaling pathway appears to protect cells from a variety of cellular stresses. On the contrary, the JNK and p38 MAPK signaling pathways have been suggested to be apoptosis-inducing pathways (Boronkai *et al.*, 2009; Rudolf *et al.*, 2008). Among the apoptosis-inducing pathways, p38 MAPK signaling pathways have been verified to be related with the apoptosis of cartilage by other researchers (Wang *et al.*, 2007). But the JNK MAPK signaling pathways have not been clarified.

SP600125 also known anthrapyrazolone or 1,9-pyrazoloanthrone is poorly soluble in water (0.0012 mg/ml in water), but was soluble in 100% DMSO. It is commonly used as highly selective inhibitors of JNK. For the selective inhibition, SP600125 inhibited JNK1, -2, and -3 isoforms with similar potency, but exhibited greater than 300-fold selectivity against related ERK1 and p38-MAPK. In cells, SP600125 significantly inhibited JNK-mediated c-Jun phosphorylation, so it is most frequently used as a small-molecule inhibitor in the JNK signaling pathway study.

Tai has showed that pretreatment with low concentrations of SNP (< 0.3 mM) for 24 hr did not cause cell injuries but could protect osteoblasts from high concentrations of SNP (> 1 mM)-induced apoptotic insults (Tai *et al.*, 2007). Their study further provides *in vitro* data to show that NO decomposed from SNP induced apoptosis of a human chondrocyte cell line (Cherng *et al.*, 2008). Investigating NO-induced chondrocyte apoptosis 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 we focused on the apoptosis of rabbit articular chondrocytes induced by SNP and investigated the apoptosis signal pathways involved in the process which SP600125 served as JNK inhibitors.

MATERIALS AND METHODS

Chondrocytes isolation and cell culture

Rabbit articular chondrocytes were harvested from cartilage slices of 3-week-old New Zealand white rabbits by enzymatic digestion as described previously (Wang *et al.*, 2007). 10^6 articular cartilage cells were seeded in culture

flasks and grown in DME/F12 medium 4 ml supplemented with 30% fetal bovine serum (Hyclone, Logan, Utah, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified 5% CO₂ as monolayers culture. Hyaluronidase, Trypsin and Collagenase type II were purchased from Sigma (St. Louis, MO, USA). Careful attention was given to maintaining the culture; cells were observed under an inverted microscope to see the daily cell growth, cells retained their round shape and displayed no morphologic changes. The medium was changed every 2 days after 72 hr for the first time interval of replacement of medium. To avoid any problems of dedifferentiation, all experiments were performed by using first generation confluent chondrocytes.

Chondrocyte stimulation with SNP and SP600125

Sodium nitroprusside (SNP) was obtained from Johnson Matthey (London, UK). SP600125 was from the Alexis Biochemicals (San Diego, CA, USA). The chondrocytes were divided into two groups and kept in serum-free DME/F-12 at 37°C for 24 hr under 5% CO₂ and then the first group was treated with various concentrations of SNP as (0 (control 1), 0.1, 0.4, 1, 2 mM) for 24 hr. The chondrocytes of the second group were treated with SNP and/or JNK inhibitor SP600125 for 24 hr as following: control 2 (complete medium containing 0.2% DMSO, which is the vehicle of SP600125), SP600125 0.1 µM, SNP+SP600125 0.1 µM, SP600125 1.0 µM, SNP+SP600125 1.0 µM, SP600125 10 µM, SNP+SP600125 10 µM, SP600125 20 µM, SNP+SP600125 20 µM. SP600125 was added 30 min before treatment with SNP (1 mM). Every experimental group had the same treatment protocol repeated at least 3 times.

Caspase-3 activity

The caspase-3 assay system was purchased from Promega (Madison, WI, USA). The activity of caspase-3 was determined as the cleavage of the colorimetric substrate by measuring the absorbance at 405 nm. Briefly, cell lysates of the first passage chondrocytes (10^6 cells/ml) treated with SNP, SP600125 and 50 µM Z-VAD-FMK inhibitor at 37°C for 24 hr were prepared as described previously. After different treatments, cells were harvested, washed three times with PBS and resuspended in Cell Lysis Buffer at a concentration of 10^8 cells/ml. The cell lysates were centrifuged at $15,000 \times g$ at 4°C for 20 min and cell extracts were collected. The level of caspase-3 enzymatic activity in the cell lysate was measured in a total volume of 100 µl in 96-well plates at least

1×10^6 cells/assay according to the manufacturer's protocol. Ac-DEVD-pNA Substrate (2 μ l) was added to all wells and absorbance was read as 405 nm after 4 hr incubation at 37°C. The enzyme activity was calculated from a standard curve prepared using pNA Standard. The relative levels of pNA were normalized against the protein concentration of each extract. The specific activity (SA) of caspase-3 was calculated via micrograms of protein in each 100 μ l sample volume which were determined by the method of Bradford using a BSA standard curve.

Flow cytometric analysis of apoptosis rate

Apoptotic cells were quantified by annexin V and PI double staining using Annexin V-FITC kit purchased from Jingmei Biotech, Ltd (Shenzhen, Guangdong, China). The cells were treated with SNP and/or SP600125, with changes of the medium on alternate days. Chondrocytes were harvested by centrifugation and washed with ice-cold PBS. 5 μ l of Annexin V-FITC (10 μ g/ml) and 10 μ l PI (20 μ g/ml) were added for 15 min in the dark before harvesting. After labeling, all cells were washed with ice-cold binding buffer (PBS) and harvested. Then the cells were resuspended in binding buffer as a concentration of 1×10^6 cells/ml, in which 100 μ l of cell suspension was added in 5 ml FCM tube. Quantitative analysis of the level of apoptosis was done by using a Flow Cytometer (BD, CA, USA). The apoptotic percentage of 10,000 cells has been determined and analysed. All the experiments reported in this study were performed three separate replicates per group.

In situ cell death detection (TUNEL) of chondrocytes

To explore the effect of SP600125 on NO-induced chondrocytes apoptosis, we used TdT-mediated dUTP nick end labeling (TUNEL) method to show the apoptotic cells. The in Situ Cell Apoptosis Detection Kit was from Sino-American biotechnology company (Luoyang, Henan, China). After exposure to SNP and/or SP600125 for 24 hr, the primary chondrocytes were replanted on coverslips, cells on coverslips were fixed with 4% paraformaldehyde for 30 min and stuck on slides with balsam and the endogenous peroxidases were blocked by 3% H_2O_2 at room temperature for 10 min. The TdT and Biotin-11-dUTP reaction were performed in a humidified chamber at 37°C for 1 hr. After washing in PBS (3 $\times$ 5 min), chondrocytes were incubated with Blocking Reagent for 30 min at room temperature, followed by Avidin-HRP at 37°C for 1 hr in a humidified chamber. Biochemical controls were made with positive control slides treated with DNase. As a negative control, slides were incubated

with PBS instead of TdT. After staining with diaminobenzidine (DAB) components the slides were observed under a light microscope ($\times$ 200). The percentage of apoptotic cells was calculated by dividing TUNEL-positive cells by the total number of cells visualized in the same field. Three independent experiments were then averaged and statistically analyzed.

Western blot analysis

After chondrocytes reached confluence, confluent original chondrocytes were induced to create the first generation. Total protein was extracted from chondrocytes using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The protein was determined using the BCA protein concentration assay kit (Pierce, Rockford, IL, USA) and protein samples were boiled for 10 min in equal volumes. Polyclonal antibodies p53, caspase-3 and NF- κ B (p65) were from Santa Cruz (Santa Cruz, CA, USA). The equal amounts of protein samples were separated by 10% SDS PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Boston, MA, USA). After transfer, the PVDF membranes were blocked by 5% non-fat milk blocking buffer (PBS-T) for 1 hr, incubated overnight with primary antibodies at 4°C, washed, and incubated with a secondary batch of antibodies at 37°C for 1 hr. After incubation with appropriate primary and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (anti-rabbit IgG 1:5,000, Cell Signaling Technology; anti-mouse IgG 1:10,000, Santa Cruz Biotech, Inc.), the PVDF membranes were reacted with enhanced chemiluminescence reagent (Pierce) and exposed to X-ray films for different time periods. β -actin antibody from Lab Vision corporation (Fremont, CA, USA) served as loading control. Densitometric analysis was performed using Work-Lab software (UVP, Montpelier, MD, USA). Densitometric summaries were expressed as ratios of sample to β -actin.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was isolated from the cultured chondrocytes using Trizol reagent (Invitrogen) according to the method of Hummon *et al.* (2007). The equal amounts of RNA (3 μ g) were transcribed to 20 μ l cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Ontario, Canada) according to the manufacturer's description. The cDNAs encoding β -actin and caspase-3 amplified from 3 μ l of the cDNA reaction mixture using specific gene primers. PCR amplification was performed using the specific gene primers

Table 1. Rabbit-specific primer sets for RT-PCR analysis

Gene	Primers Sequence	Product size (bp)
caspase-3	Forward: 5-CACGGTGATGAAGGAGTC-3 Reverse: 5-GCAAGCCTGAATAATGAA-3	129bp
β -actin	Forward: 5-ACACTGTGCCCATCTACGAGG-3 Reverse: 5-TCGGCTGTGGTCACGAAGGAGT-3	133bp

(append to the Table 1). PCR was performed with Platinum Taq polymerase (Invitrogen) under the following conditions: initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, 45.5°C (β -actin and caspase-3) for 1 min, extension at 72°C for 1 min followed by 10 min at 72°C.

The PCR products were measured by 2% agarose gels and stained with ethidium bromide. The densities of each band were analyzed using Work-Lab software (UVP). The expression of β -actin was used as an internal control for the analysis of a constitutively expressed gene.

Statistical analysis

The results were expressed as the mean $\pm$ S.D. and analyzed by SPSS13.0 for Windows. The difference between the treated and untreated groups (control) was determined by one-way ANOVA (Newman-Keuls post hoc test) or Chi-square test. Statistical significance was set at $P < 0.05$.

RESULTS

JNK inhibitor SP600125 can reduce SNP-induced caspase-3 activity

Caspase-3 activation is considered a convenient marker of apoptosis and is regarded as the point of no return in the proapoptotic signaling cascade. The analysis of specific caspase-3 activation showed that exposure of chondrocytes to inhibitor of JNK (SP600125) leads to a large decrease in the activity of caspase-3. Treatment with 0.1, 0.4, 1.0 or 2.0 mM SNP produced a concentration-dependent increase in caspase-3 activity, however, this increase was inhibited by JNK inhibitor, SP600125 (Fig. 1). This indicates that SP600125 overexpression prevents upregulation of the activity of caspase-3 in response to SNP.

SP600125 inhibits the apoptosis of primary cultured rabbit chondrocytes induced by SNP

In order to examine the functional role of SP600125 in apoptotic cell death, rabbit chondrocytes were exposed to SNP of different concentrations, and were then treat-

Fig. 1. JNK inhibitor, SP600125 can down-regulate the activity of caspase-3 induced by SNP. Rabbit cartilage chondrocytes were treated to NO donor SNP of various concentrations at 37°C for 24 hr (A). Chondrocytes were exposed to DMSO (SP600125 vehicle, control) or pretreated with JNK inhibitor SP600125 for 30 min and followed by stimulation with SNP for 24 hr (B). The activity of caspase-3 from cell lysates was measured using the capase-3 assay kit.

SNP-induced apoptosis and JNK signaling pathways

Fig. 2. The effect of JNK inhibitor SP600125 on the rate of SNP-induced chondrocytes apoptosis. Rabbit cartilage chondrocytes in a serum-free medium were exposed to NO donor SNP of various concentrations for 24 hr (001-005 respectively). Chondrocytes were exposed to DMSO (SP600125 vehicle, control, 006) or pretreated with SP600125 for 30 min and followed by stimulation with SNP for 24 hr (007-014 respectively). The rate of chondrocytes apoptosis was detected by FCM assay, and the representative scattergram of experiments was shown in Fig. 2A. A two-parameter cytogram of log (FL1) (annexin) vs. log (FL3) (PI) was drawn. The apoptotic cells (annexin V⁺/PI⁺) were detected in the right lower quadrant and the data was expressed as mean $\pm$ S.D. of experiments. The relation between the apoptosis rate and the concentration of SNP was shown in (B). The difference among the different concentration of SNP and SP600125 was in (C). * indicates a significant difference from the control ($P < 0.05$). # $P > 0.05$ vs control.

ed with JNK inhibitor, SP600125, and then analyzed by Annexin V-FITC/PI FCM (Figs. 2A, C). Mechanical dead cells (AnnexinV-FITC $-/PI^{+}$) was shown in the upper left quadrant; early apoptotic cells (AnnexinV-FITC $+/PI^{-}$) in the lower right quadrant; late apoptotic cells (AnnexinV-FITC $+/PI^{+}$), in the upper right quadrant; normal cells (AnnexinV-FITC $-/PI^{-}$) in the lower left quadrant. We found that the control group had more than 90% of intact, living cells and only less than 10% of cells in the early and late phases of apoptosis (Fig. 2A). The flow cytometric figure was shown in Fig. 2A using this method. The positive percentage in Fig. 2B, shows that compared with untreated control, different concentration of SNP could increase the apoptosis rate of chondrocytes in a dose-dependent manner ($P < 0.05$). And from Fig. 2C we found that SP600125 was able to decrease the apoptosis rate induced by SNP, and could partially block the apoptosis of chondrocytes induced by SNP, compared with untreated control ($P < 0.05$).

The effect of SP600125 on NO-induced chondrocytes apoptosis

The TUNEL assay was performed to identify the effect

of SP600125 on NO-treated chondrocytes. The results show that the TUNEL positive cells consistently exhibited nuclear condensation with a subset *also* exhibiting nuclear “blebbing”, both hallmarks of apoptosis. When the concentration of NO donor SNP was increased, more and more apoptotic cells appear. With the addition of JNK inhibitor, SP600125, evidence of reduction of positive cells was found according to the TUNEL assay. It indicated that SP600125 could partially block chondrocyte DNA strand fragmentation and further apoptosis induced by NO (Fig. 3).

Effects of SNP on NF- κ B p65, p53 and caspase-3 protein expression

To examine the chondrocytes apoptosis induced by SNP, cells were stimulated with indicated SNP for 24 hr in serum-free medium. The total protein was extracted and analyzed for the levels of NF- κ B p65, p53 and caspase-3 by Western blotting with the respective antibody. From the result of Western blotting, we found NF- κ B p65, p53 and caspase-3 protein expression up-regulated in the group treated with SNP as the concentration of SNP increasing, compared with the untreated control

Fig. 3. DNA strand fragmentation demonstrated by TUNEL assay. Chondrocytes were exposed to NO donor SNP and/or JNK inhibitor SP600125 for 24 hr as previously described. The positive nuclei (apoptosis) were stained as brown by the TUNEL reaction, and the negative nuclei were blue. Magnification: $\times 200$.

($P < 0.05$) (Fig. 4). So we can infer that SNP can activate NF- κ B p65, p53 and caspase-3 protein expression.

Effects of JNK inhibitor SP600125 on NF- κ B p65, p53 and caspase-3 protein expression

To investigate the effects of SP600125 on protein expression, the chondrocytes were incubated in serum-free medium (DMSO or SP600125) for 30 min before treatment with/without SNP for 24 hr. Western blotting analysis demonstrated that protein expressions of NF- κ B p65, p53 and caspase-3 were significantly higher in group

treated with SNP as compared with DMSO control, but the protein expressions was down-regulated when the concentration of SP600125 increasing, compared with group treated with 0.1 mM SNP ($P < 0.05$). However, even when the concentration of SP600125 increasing, there was no significant difference among the group without stimulation with SNP, compared with the DMSO control ($P > 0.05$) (Fig. 4). Thus the results indicated that NF- κ B p65, p53 and caspase-3 proteins were involved in JNK pathway.

Fig. 4. JNK inhibitor, SP600125 can inhibit protein expressions of NF- κ B p65, p53 and caspase-3 which were increased by SNP. Rabbit cartilage chondrocytes in serum-free medium were exposed to indicated concentration of NO donor SNP (A, B) for 24 hr, and DMSO (SP600125 vehicle, control) or pretreated with SP600125 for 30 min then followed by treatment of SNP (A, C) for 24 hr. The total protein subjected to PAGE and Western blot analysis with respective antibodies against NF- κ B p65, caspase-3 or p53, followed by incubation with anti-rabbit secondary antibodies and revelation by enhanced chemiluminescence. The protein expressions of p65, p53, caspase-3 and β -actin are shown (A). The mean densitometric value of each sample protein divided by β -actin from three independent experiments was depicted as bar graphs (B, C). * $P < 0.05$ vs control. # $P > 0.05$ vs control.

JNK inhibitor reduces SNP-induced caspase-3 mRNA expression

To examine whether caspase-3 mRNA was involved in SNP-induced chondrocyte apoptosis, we assessed the caspase-3 mRNA expression using RT-PCR. SNP induces caspase-3 mRNA expression compared with normal control ($P < 0.05$). However, this increase was inhibited by SP600125 compared with normal control ($P < 0.05$). There was no significant difference without stimulation with SNP, compared with the normal control ($P > 0.05$) (Fig. 5).

DISCUSSION

During inflammation, reactive oxygen species can be overproduced by chondrocytes and induces cell injuries. NO radical is one of the important reactive oxygen species. NO decomposed from SNP caused apoptosis to human chondrocytes via an apoptotic pathway (Cherng *et al.*, 2008). Apoptosis is an indispensable and continuously occurring process that eliminates obsolete or damaged cells, whereas uncontrolled apoptosis culminates in various diseases. The induction of apoptosis in response to various agonists is dependent on the signal strength and promoting or antagonizing signaling pathways. NO-induced apoptosis has been shown for macrophages, neurons, and thymocytes (Chang *et al.*, 2010; Nishikawa *et al.*, 2007; Migita *et al.*, 2003). The generation of NO in chondrocytes is primarily caused by inflammatory cytokines. It is one of the leading causes of the chondrocytes programmed cell death (Nakagawa *et al.*, 2010). It has previously been shown that JNK pathway mediates

NO-induced apoptosis (Cherng *et al.*, 2008). However, little is known about the existence of NO-induced JNK-c-Jun signaling and subsequent gene regulation in apoptosis. This study has investigated which factors are involved in NO-induced chondrocytes apoptosis in JNK pathways.

In this experiment, we noticed that NO would induce chondrocyte apoptosis in a dose-dependent manner through morphologic and biochemical evidences of apoptosis (TUNEL and Annexin V-FITC/PI FCM), and this effect could be inhibited by JNK-specific inhibitor SP600125. Annexin V and Propidium iodide staining is a conventional method to detect apoptosis in cultured cells including chondrocytes (D'Lima *et al.*, 2004). The exact mechanism of NO leading to cells undergoing apoptosis has not been clarified. One possible explanation is that the reaction of NO with oxygen radicals results in the formation of peroxynitrite (Heck *et al.*, 2005). Peroxynitrite, as the upstream trigger of JNK (Clancy *et al.*, 2001), may attack aromatic amines such as pyridine and purine, finally leading to activation of caspase-3, DNA fragmentation, and apoptosis.

Apoptosis of chondrocytes characterizes a number of diseases involving cartilage degeneration shown in animal models (Chen *et al.*, 2005). The involvement of JNK has mainly been shown in animal models of inflammatory articular diseases (Ea *et al.*, 2005). Inhibitors of both p38 and JNK inhibit cell death and thus serve as therapeutic agents in animal models of chondrocyte death (Niemenen *et al.*, 2005). Our present results are in accordance with previous studies, demonstrating that the JNK pathways have a NO induced activation, and thus, are most proba-

Fig. 5. JNK inhibitor, SP600125 can suppress caspase-3 mRNA expression which was increased by SNP. Confluent cultures of rabbit cartilage chondrocytes in serum-free medium for 24 h were exposed to indicate concentration of NO donor SNP, and DMSO (SP600125 vehicle, control) or pretreated with SP600125 for 30 min then followed by treatment of SNP for 24 hr. The total RNA extracts were subjected to RT-PCR analysis with caspase-3 primer. The mean densitometric value of the sample divided by β-actin from experiments was depicted as bar graphs (A, B). * Different from control, $P < 0.05$. # $P > 0.05$ vs control.

bly involved in the observed NO stress-induced chondrocyte apoptosis. All these findings together indicate that JNK may participate in the induction of the catabolic program in cartilage.

There is strong evidence that a sustained activation of JNK precedes apoptosis (Zhang *et al.*, 2004). Cells can be protected from apoptosis by blocking JNK pathway (Liu *et al.*, 2002). It strongly suggested that JNK activation is necessary for apoptosis. Although the exact role of JNK in these processes is yet to be elucidated, it is accepted that members of the MAPK kinases (MKKs) activate JNK by dual phosphorylation (Fleming *et al.*, 2000). JNK further phosphorylated of c-Jun on sites Ser-63 and Ser-73 (Xu *et al.*, 2010). c-Jun is a specific and important functional endpoint of JNK signaling. SP600125 is a reversible ATP competitive inhibitor, exhibits an over 300-fold greater selectivity for JNK as compared to ERK1 and p38. SP600125 dose-dependently inhibits the phosphorylation of c-Jun, thus inhibits phosphorylation of JNK target proteins including JNK itself (Bennett *et al.*, 2001). In order to determine whether activation of JNK is directly associated with the chondrocyte apoptosis, we sought to block JNK activity using SP600125 and to determine the effect on the extent of apoptosis induced by SNP. Our results showed that inhibition of the JNK pathway with SP600125 attenuated the effects of NO induced apoptosis and caspase-3 activation.

NF- κ B is a member of the Rel family including p50 (NF- κ B 1), p52 (NF- κ B 2), Rel A (p65), c-Rel, Rel B, and Drosophila morphogen dorsal gene product (Siebenlist *et al.*, 1994). During the regulation of apoptosis the functional relationship between NF- κ B and p53 is controversial. NF- κ B appears to function as either an upstream or downstream signaling molecule of p53 (Zhang *et al.*, 2001). Recent studies reported that NO-induced activation of p38 kinase activates NF- κ B, which led to increased p53 expression (Kim *et al.*, 2002b). This suggested that NF- κ B may be an upstream signaling molecule of p53 during the apoptotic process. In the current study, we found from results of Western blotting that SP600125 reduced NO-activated NF- κ B p65 expression. So we propose that NF- κ B might be involved in a JNK MAPK pathway.

The p53 tumor suppressor protein plays a critical role in the cellular response to various cellular stresses (Levine, 1997). Recent studies demonstrated that JNK activation can phosphorylate p53 at serine 15 (Fogarty *et al.*, 2003). This indicated that JNK mediates phosphorylation of the p53 tumor suppressor gene, which in turn modulates cell proliferation, differentiation, and apoptosis (She *et al.*, 2000). The present study revealed that

NO activated NF- κ B p65 and p53, and the activation can be inhibited by SP600125. So we assume that JNK is the upstream activator of NF- κ B, which could lead to p53 stimulation.

Caspases play a central role in the apoptosis machinery. They participate in a cascade that is triggered in response to pro-apoptotic signals and culminates in cleavage of a set of target proteins, resulting in a disassembly of the cell (Said *et al.*, 2004). Based on caspase-3 activity determinations as an apoptotic marker, all existing data indicates that caspase activation is a requirement for chondrocyte apoptosis. From caspase-3 activity assay and protein expression with Western blotting, we have obtained consistent results that SNP dose-dependently increased caspase-3, however, this increase was inhibited by SP600125. It may be indicated that caspase-3 may be requirement for JNK pathway, and JNK activation may be the upstream of caspase-3.

By measuring caspase-3 activation, we found the correlation of JNK activation and the rate of apoptosis. Conclusively, the JNK pathway is an important and early signal transducing mechanism in NO-induced apoptosis in rabbit chondrocyte.

NO has been reported as the primary inducer of apoptosis in multiple pathways which are involved in both the initiation and execution of chondrocyte apoptosis (Wang *et al.*, 2007). Our previous study showed that p38 MAPK pathway is necessary for NO-induced apoptosis in chondrocytes. Recent study suggests that doxorubicin induces cardiac apoptosis via the activation of JNK-p38 and p53-mediated signalling pathway (Kim *et al.*, 2011). So we infer that apoptosis in rabbit chondrocytes through JNK pathway.

In conclusion, the inflammatory mediator NO can induce apoptosis in rabbit chondrocytes through JNK activation. Our present study shows that NO-induced activation of JNK activates NF- κ B, which leads to the increased transcriptional expression of p53. NO stress induced apoptotic cell death in chondrocytes as revealed by Annexin Vand propidium iodide double-staining and flow cytometry as well as by activation of caspase-3. The increased expression and accumulation of p53 causes apoptosis by inducing expression and activation of caspase-3. Further, we show that SP600125 can block this pathway and NO-induced apoptosis. Our results may help to further elucidate the pathomechanism in conditions that involve NO such as OA and RA.

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