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SP600125 blocks the proteolysis of cytoskeletal proteins in apoptosis induced by gas signaling molecule (NO) via decreasing the activation of caspase-3 in rabbit chondrocytes

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ABSTRACT

NO plays a key role in the pathological mechanisms of articular diseases. As cytoskeletal proteins are responsible for the polymerization, stabilization, and dynamics of the cytoskeleton network, we investigated whether cytoskeletal proteins are the intracellular pathological targets of NO. We aimed at clarifying whether the cytoskeleton perturbations involved in apoptosis are induced in rabbit articular chondrocytes by NO, which can be liberated by sodium nitroprusside (SNP) treatment. The first passage rabbit articular chondrocytes were cultured as monolayer for the experiments, and the effects of NO were tested in the presence of JNK-specific inhibitor, SP600125. SNP treatment of cultured chondrocytes caused significant apoptosis in a concentration-dependent manner (time and dose), as evaluated by TUNEL assay and Annexin V flow cytometry, while the apoptosis was reduced by the SP600125 addition 30 min before SNP treatment. Besides, SP600125 decreased significantly the protein expression of total caspase-3 and the intracellular gene expression of caspase-3, measured by Western blot analysis and PCR. SP600125 also increased the cytoskeletal protein expressions. These results suggested that JNK pathway plays a critical role in the NO-induced chondrocyte apoptosis, and SP600125 treatment blocks the dissolution of the cytoskeletal proteins via activation of caspase-3 pathways.

1. Introduction

Nitric oxide (NO), an important gaseous free radical, is synthesized from L-arginine by inducible nitric oxide synthase (iNOS). As an inter- and intracellular pleiotropic signaling molecule in many cell types, including articular chondrocytes, the effects of NO are extremely rapid, local, and potentially toxic (Li et al., 2010). Excess of NO formation is a hallmark of cartilage degradation in diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) (Stradner et al., 2008). Among OA cases, chondrocytes' apoptosis is far more often observed in normal subjects (Yang and Lee, 2010). OA involves progressive destruction of the cartilage matrix due to a pathological imbalance of chondrocyte functions (Kim and Blanco, 2007). Chondrocytes are the only cell type in articular cartilage and play an essential role in keeping cartilage integrity. Excessive production of NO locally causes the chondrocytes to go apoptosis and cell death, leading to pathological changes in OA and RA.

Apoptosis is mediated by mitogen-activated protein kinases (MAPKs), and it contributes to the chondrocyte loss and subsequent cartilage degeneration (Schlezinger et al., 2006). MAPKs are important mediators of intracellular signals during various biological events involved in development, proliferation, differentiation, and apoptosis (Zhang et al., 2013). In mammalian cells, MAPKs are comprised of three principal family members: the extracellular signal-regulated kinases (ERK), the p38 MAPK, and the c-Jun NH2-terminal kinase (JNK) (Mahalingam et al., 2013). The p38 MAPK and JNK cascades appear to be mainly involved in cellular stress responses (Mo et al., 2012; Tarapore et al., 2013). Recent evidence implicates that JNK plays an important role in proapoptotic pathways (Tarapore et al., 2013).

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Caspases, a family of cysteine proteases, play a central role in many aspects of the apoptotic pathway. Based on the sequence similarity among the protease domains, caspases are divided into three groups: 1) inflammatory caspases including caspase-1, −4, −5, −11, −12, −13, and −14; 2) effector caspases including caspase-3, −6, and −7; and 3) initiator caspases including caspase-2, −8, −9, and −10 (Nadiri et al., 2006). It is unclear yet whether all of these proteases take part in apoptosis, but caspase-3 is believed to be involved in propagating the caspase cascade, and be activated at the execution phase of programmed cell death (Inoue et al., 2009). In addition, caspase-3 has long been considered as the important mediator in chondrocyte apoptosis induced by NO (Ueng et al., 2013).

The chondrocyte cytoskeleton, a three-dimensional (3D) network, provides the cell with its mechanical integrity, and has been linked to the process of chondrocyte mechanotransduction through which cartilage cells sense and adapt to external mechanical stimuli (Campbell et al., 2007). An intact cytoskeleton is essential for the chondrocyte homeostasis, and a deregulated cytoskeleton leads to abnormal signaling and a catabolic phenotype (Blain, 2009). Nitric oxide (NO), a multifunctional reactive oxygen species (ROS), also appears to simulate JNK, leading to the disruption of the cytoskeleton in the apoptosis (Malerba et al., 2008).

Chondrocyte phenotype is affected by cell shape indirectly, and in fact it is regulated by actin cytoskeleton (Nurminsky et al., 2007). Further studies have confirmed the importance of actin organization in controlling the chondrocyte phenotype. However, to date, the molecular mechanisms responsible for the interrelationship of chondrocyte differentiation and actin organization are largely unknown, and surprisingly little is known about the chondrocyte-specific intracellular regulators of cytoskeletal rearrangement.

The purpose of this study was to investigate the disruption of the cytoskeleton during NO-induced cell death in rabbit articular chondrocytes. We demonstrated that NO induced apoptosis through stimulating caspase-3 activation JNK-specific inhibitor, SP600125, blocked the dissolution of the cytoskeletal proteins during the process of apoptosis.

2. Materials and methods

2.1. Materials and reagents

Hyaluronidase, collagenase type 2 and trypsin were purchased from Sigma (St. Louis, MO, USA). DME/F-12 was from Hyclone (Logan, UT, USA). Sodium nitroprusside (SNP) was obtained from Johnson Matthey Co. (Royston, UK), and JNK inhibitor SP600125 [Anthra(1,9-cd)pyrazol-6(2H)-one;1,9-pyrazoloanthrone] was purchased form the Alexis Biochemicals (Lausen, Switzerland). A colorimetric caspase-3 assay system was purchased from Promega (Madison, WI, USA), and the Annexin V-FITC kit was from Jingmei Biotech Co., Ltd (Shen Zhen, Guangdong, China). In Situ Cell Apoptosis Detection Kit was obtained from Sino-America Biotechnology Company (Luo Yang, Henan, China), while the Trizol Reagent was from Invitrogen Life Technologies (Carlsbad, California, USA). The BCA protein concentration assay system and rabbit anti-caspase-3, anti-actin and anti-tubulin antibodies were purchased from Santa Cruz Biotech, Inc (Santa Cruz, CA, USA). The rabbit anti-vimentin antibody was from NeoMarkers, Inc (Fremont, CA, USA), while the rabbit anti-β-actin antibody was from Lab Vision corporation (Fremont, CA, USA). Horseradish peroxidase-conjugated anti-rabbit secondary antibodies were bought from Santa Cruz Biotech, Inc. RevertAid First Strand cDNA Synthesis Kit was from Fermentas Life Sciences (Fermentas, Habover, MD, USA), and all the primers in RT-PCR were synthesized by Beijing Sunbiotech Co., Ltd (Beijing, China). PVDF membranes were purchased from Millipore (Billerica, MA, USA). SuperSignal West Pico Chemiluminescent (ECL) Western blot detection system was obtained from Pierce Biotech Inc. (Pierce, Rockford, IL, USA). All other reagents were obtained from commercial sources. All solutions were prepared using three times distilled water.

2.2. The isolation of chondrocytes and treated with SNP or SP600125

Normal rabbit articular cartilage was obtained from knee joints of a 3-week-old New Zealand white rabbit by enzymatic digestion. This study was approved by the Ethics Committee of Xi'an Jiaotong University Health Science Center (license number 2011058). The chondrocytes were obtained by sequential digestions using hyaluronidase, trypsin and collagenase as described previously (Wang et al., 2007). The isolated cells were collected and seeded in culture flasks, and grown in 4 ml DME/F12 medium supplemented with 30% (v/v) fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified 5% CO_2 incubator at 37 °C as monolayers culture. The medium was changed every 2 days, and cells reached confluence by day 7–10. To avoid the dedifferentiation, all experiments were performed on first generation confluent chondrocytes. All cells were kept in serum-free DME/F-12 for 24 h at 37 °C under 5% CO_2. Then different concentrations [0, 0.1, 0.4, 1, 2 mM] of SNP were added in the medium of first group for 24 h. Then the concentration of 1 mM of SNP was selected as the follow-up test concentration, based on the results of flow cytometry and TUNEL analysis. Then the cells were treated with SP600125 alone or with SP600125 + SNP for 24 h as follows: control (only containing 0.2% DMSO), 0.1 μM SP600125, 1 mM SNP + .1 μM SP600125, 1 μM SP600125, 1 mM SNP + 1 μM SP600125, 10 μM SP600125, 1 mM SNP + 10 μM SP600125, 20 μM SP600125, and 1 mM SNP + 20 μM SP600125. SP600125 was added into the cells 30 min before treatment with SNP.

2.3. Caspase-3 activity test

Caspase-3 activity was determined by using Caspase-3 Assay System, a Colorimetric kit. The cells were harvested by centrifugation at 4 °C, 400 g for 10 min, and washed with ice-cold PBS and resuspended in Cell Lysis Buffer at a concentration of 10^8 cells/ml. The cells were lysed and the supernatant fraction (cell extract) was collected. Caspase-3 enzymatic activity of cell extracts was measured in a total volume of 100 μl in 96-well plates. The chondrocyte density was adjusted to 10^6 cells/ml and then 2 μl of caspase-3 substrate was added and the cells were incubated at 37 °C for 4 h. The substrate was labeled p-nitroanilide (pNA): when substrate was recognized and cleaved by the caspase-3, pNA was released. Caspase-3 activity was calculated by measuring the amount of free pNA at a wavelength of 405 nm with an enzyme-linked immunosorbent assay reader (BMG, Germany). The enzyme activity was calculated by using a standard curve provided by pNA standard solutions.

2.4. Apoptosis rate detected by Annexin V-FITC/propidium iodide flow cytometry

To determine the apoptosis rate, the Annexin V-FITC kit was used to determine the early and late apoptotic activities according to the manufacturer's protocol. After SNP and/or SP600125 administrations, the cells were harvested, washed with 1 × ice-cold PBS and re-suspended in 100 μl binding buffer at a concentration of 1 × 10^6 cells/ml. A total of 5 μl of Annexin V-FITC and 10 μl of 20 μg/ml propidium iodide (PI) were added and the mixture was incubated for 15 min in the dark. Finally, 400 μl of binding buffer was added to the cells and the mixture was analyzed with a flow cytometer (BD, CA, USA). The apoptotic percentage of 1 × 10^4 cells was determined, and all the experiments reported in this study were performed three times for the sake of statistical analysis.

2.5. Examination of the apoptosis of chondrocyte by TUNEL

To examine the effect of SP600125 on apoptosis, the cells were