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Original Contribution

Dominant roles of Fenton reaction in sodium nitroprusside-induced chondrocyte apoptosis

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ABSTRACT

Sodium nitroprusside (SNP) has been widely used as an exogenous nitric oxide (NO) donor to explore the molecular mechanism of NO-mediated chondrocyte apoptosis during the latest two decades. We have recently found that NO-independent ROS play a key role in SNP-induced apoptosis in rabbit chondrocytes. This study aims to investigate what kind of ROS and how the reliable ROS mediators mediate the SNP-induced apoptosis. Data shows that SNP and NO-exhausted SNP (SNPex) induced ROS production or cytotoxicity to identically degree. SNP induced a marked increase in iron ions, superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) level. H_2O_2 scavenger (CAT) and $^{\bullet}OH$ scavenger (DMSO) significantly inhibited SNP-induced chondrocyte apoptosis. Iron ions chelator (DFO) entirely prevented SNP-induced chondrocyte apoptosis. In contrast, $O_2^{\bullet-}$ scavenger (SOD) and glutathione depletion agent (BSO) promoted SNP-induced cytotoxicity. $K_3[Fe(CN)_6]$ exhibited no cytotoxicity, and H_2O_2 alone up to 250 μM or iron ions alone up to 90 μM is non-cytotoxic to chondrocytes. Combination of 25 μM $FeSO_4$ and 100 μM H_2O_2 in the presence of BSO induced chondrocyte death similar to SNP treatment. Fetal bovine serum (FBS) enhanced iron ions release from SNP and the cytotoxicity of SNP. Our data shows that the extracellular Fenton reaction between iron ions released from SNP and H_2O_2 induced by SNP plays a key role in SNP-induced chondrocyte apoptosis. Overall, our results indicate that the potential of SNP to increase iron ions and ROS should be especially considered for some biological functions and, possibly, also for clinical applications of this drug.

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1. Introduction

Sodium nitroprusside (SNP), a water-soluble iron nitrosyl complex clinically used as a powerful vasodilator for treatment of hypertension, has been used to investigate the cytotoxic effects of nitrosative stress in basic study. Since Blanco and co-workers for the first time concluded that NO released from SNP dominated SNP-induced chondrocyte apoptosis [1,2], SNP has been widely used as an exogenous NO donor to explore the molecular mechanism of NO-mediated chondrocyte apoptosis during the latest two decades [3–30]. However, more potent NO donor including S-nitroso-*N*-acetyl-L-penicillamine (SNAP), diazeniumdiolates (NOC-5/12) as well as 3-morpholinosydnonimine (SIN-1) did not induce more apoptosis than SNP [31–33]. Moreover, the scavengers of both NO and peroxynitrite (ONOO−) had no protective effect on SNP-induced cytotoxicity in osteoarthritic synoviocytes [34]. We recently also found that although NO scavengers significantly inhibited SNP-induced NO production, they did not exhibit protective effect on SNP-induced rabbit chondrocyte apoptosis [35].

There is an ever-increasing amount of evidences showing the important roles of ROS in SNP-induced chondrocyte apoptosis [6,29,30]. Our recent studies definitely demonstrated that ROS instead of NO played a key role in SNP-induced chondrocyte death, which was further verified by our finding that resveratrol largely prevented SNP-induced chondrocyte apoptosis via scavenging ROS instead of NO [35]. Superoxide anion ($O_2^{\bullet-}$), the product of a one-electron reduction of oxygen, is the precursor of most ROS [36]. It is reported that $O_2^{\bullet-}$ is generally poorly reactive and only can attack a few molecules [36,37]. Moreover, dismutation of $O_2^{\bullet-}$ produce H_2O_2, a weaker oxidizing agent. Although H_2O_2 on its own

Abbreviations: BSO, L-Buthionine-sulfoximine; CAT, catalase; DFO, deferoxamine mesylate salt; DHE, dihydroethidium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; GSH, glutathione; NAC, N-acetyl cysteine; NOC-5, 3-(Aminopropyl)−1-hydroxy-3-isopropyl-2-oxo-1-triazene; ROS, reactive oxygen species; SNP, sodium nitroprusside; SNPex, nitric oxide exhausted SNP; SOD, superoxide dismutase

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is not particularly toxic, there are numerous examples of damage to biological molecules, in which H_2O_2 and iron are implicated [38]. The reaction between iron and H_2O_2, referred to as the Fenton reaction, is capable of generating both $^\bullet OH$, one of the strongest oxidants in nature, and higher oxidation states of iron. These oxidizing intermediates of Fenton reaction cause damage to biomolecules and play a major role in the aging process and a variety of diseases.

This study is designed to explore the reliable ROS mediators mediating the cytotoxicity of SNP in rabbit articular chondrocytes. Our findings for the first time demonstrate that the extracellular Fenton reaction between free iron ions released from SNP and H_2O_2 induced by SNP plays a key role in SNP-induced chondrocyte apoptosis.

2. Material and methods

2.1. Materials

SNP, dimethyl sulphoxide (DMSO), 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), Carboxy-PTIO (PTIO), N-acetyl cysteine (NAC), Deferoxamine mesylate salt (DFO), L-Buthionine-sulfoximine (BSO), catalase (CAT) and Rhodamine 123 (Rho 123) were from Sigma (St. Louis, USA). 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5) was from Enzo Life Sciences Inc. (New York, USA). Potassium ferricyanide (III) was from Aladdin (Shanghai, China). Ferric sulfate [$Fe_2(SO_4)_3$], ferrous sulfate ($FeSO_4$), ferrous chloride ($FeCl_2$), NaOH and hydrogen peroxide (H_2O_2) were from Guangzhou chemical reagent factory (Guangzhou, China). Dulbecco's modified Eagle medium (DMEM) was from Gibco (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Sijiqing (Hangzhou, China). 3-Amino, 4-aminomethyl-2′,7′-difluorescein, diacetate (DAF-FM DA), Dihydroethidium (DHE), $NaNO_2$, RIPA lysis reagent and superoxide dismutase (SOD) were from Beyotime Institute Biotechnology (Jiangsu, China). Trypsin and type II collagenase were from Invitrogen (California, USA).

2.2. Cell isolation and culture

Articular chondrocytes for primary culture were harvested from slices of shoulder, knee and hip-joint cartilage from 6-week-old New Zealand white rabbits as described previously [35]. Chondrocytes were isolated by enzymatic digestion of 0.25% Trypsin in PBS for 1 h and 0.2% type II collagenase in DMEM for 4–6 h. After collection by centrifugation, chondrocytes were re-suspended in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), and then seeded in culture flasks at 37 °C in humidified 5% CO_2 as monolayer culture. The primary cells were sub-cultured to generation 2 that were cultured in DMEM supplemented with 10% FBS and antibiotics for at least 24 h.

2.3. Treatments

The SNP powder was freshly dissolved in ultrapure water to obtain 100 mM concentration SNP solution before experiment. Then, SNP was diluted in water or culture medium and added to cells to obtain various concentrations. All SNP experiments were performed in dark. Exhausted SNP (SNPex) was obtained by leaving the solution of SNP under light exposure for 24 h at room temperature. NOC-5 was freshly dissolved in 0.1 M NaOH. CAT, NAC, $NaNO_2$, $Fe_2(SO_4)_3$, $FeSO_4$ and $FeCl_2$ were prepared just before the experiments by dissolving the powders in ultrapure water. SOD, DFO and BSO were dissolved in ultrapure water as stock solution and stored at −20 °C in the dark. PTIO was dissolved in DMSO, and the final concentration of DMSO was less than 0.1% (v/v) in experiment. Rabbit articular chondrocytes were pretreated with BSO for 12 h, NAC or PTIO for 2 h, and CAT, SOD, DFO, DMSO for 30 min, and then co-treated with 1.5 mM SNP for 6 h. Chondrcoytes were exposed to $NaNO_2$, NOC-5, $K_3[Fe(CN)_6]$, $Fe_2(SO_4)_3$, $FeSO_4$, $FeCl_2$ and H_2O_2 for 6 h. For all cell experiments, before drugs treatment, cells were replaced with fresh medium.

2.4. Measurement of nitrite and nitrate

NO released from SNP was indirectly quantified by measuring the oxidation by-products nitrite/nitrate. 1.5 mM SNP in water were prepared and kept in dark or exposed to sunlight at room temperature for indicated times, 50 μl of solution was then collected for nitrite/nitrate detection. Chondrocytes (1×10^6) cultured in 6-well plates at 37 °C for 24 h were subjected to the indicated treatments, then 50 μl of cell medium were collected for nitrite/nitrate measurement. Briefly, collected water or cell medium was reacted with an equal volume of Griess reagents (1% sulfanilamide, 0.1% N-[1-naphthyl] ethylenediamine dihydrochloride and 2.5% phosphoric acid) in 96-well plates. The absorbance variation was measured at a wavelength of 540 nm using an auto-microplate reader (Infinite M200, Tecan, Austria), and the nitrite concentration was determined using a curve calibrated using an identical protocol.

2.5. Cell viability and apoptosis assay

Cells were plated in 96-well plates (1×10^4 per well) for 24 h at 37 °C were subjected to the indicated treatments. Cell viability was detected by Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) using the auto-microplate reader as described previously [39]. Chondrocytes were cultured in 6-well plates were subjected to the indicated treatments, cell apoptosis was quantified by FCM (FACSCCanto II, BD Biosciences) analysis using Annexin V-FITC/PI apoptosis detection kit (Bestbio, Shanghai, China) according to the manufacturer's protocol as described previously [39]. Chondrcoytes cultured in 6-well plates for 24 h were subjected to the indicated treatments, and then cell morphological changes were recorded using a digital camera (Sony, Japan) after treatment with drugs for the time indicated by an inverted microscope. Original magnification: 100× (Carl Zeiss, Germany).

2.6. Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

Chondrcoytes cultured in 6-well plates for 24 h were subjected to the indicated treatments. Loss of $\Delta\Psi m$ was analyzed by FCM assay with Rho 123 staining as previously described [39]. Briefly, cells were harvested and stained with 5 μM Rho 123 at 37 °C for 20 min in dark, and then washed with PBS twice and subsequently assayed by FCM.

2.7. Measurement of intracellular ROS and NO

Chondrcoytes cultured in 6-well plates for 24 h were subjected to the indicated treatments. Intracellular ROS or NO was quantified by FCM analysis with DCFH-DA or DAF-FM DA staining which is cell-permeable as described previously [35]. The oxidation sensitive probe DCFH-DA is cleaved by nonspecific esterases and converts to highly fluorescent DCF upon oxidation by ROS. The NO sensitive probe DAF-FM DA is cleaved by nonspecific esterases and converts to intensely fluorescent benzotriazole derivative upon oxidation by NO. Chondrocytes were harvested and stained with 20 μM DCFH-DA for 30 min or with 5 μM DAF-FM DA for 20 min at 37 °C in the dark, re-washed with PBS three times before analyzed quantitatively using FCM.

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