p38 Kinase Regulates Nitric Oxide-induced Dedifferentiation and Cyclooxygenase-2 Expression of Articular Chondrocytes

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ABSTRACT

Background: Caveolin, a family of integral membrane proteins are a principal component of caveolae membranes. In this study, we investigated the effect of p38 kinase on differentiation and on inflammatory responses in sodium nitroprusside (SNP)-treated chondrocytes. **Methods:** Rabbit articular chondrocytes were prepared from cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion. SNP was used as a nitric oxide (NO) donor. In this experiments measuring SNP dose response, primary chondrocytes were treated with various concentrations of SNP for 24 h. The time course of the SNP response was determined by incubating cells with 1 mM SNP for the indicated time period (0～24 h). The cyclooxygenase-2 (COX-2) and type II collagen expression levels were determined by immunoblot analysis, and prostaglandin E_2 (PGE_2) assay was used to measure the COX-2 activity. The tyrosine phosphorylation of caveolin-1 was determined by immunoblot analysis and immunostaining. **Results:** SNP treatment stimulated tyrosine phosphorylation of caveolin-1 and activation of p38 kinase. SNP additionally caused dedifferentiation and inflammatory response. We showed previously that SNP treatment stimulated activation of p38 kinase and ERK-1/-2. Inhibition of p38 kinase with SB203580 reduced caveolin-1 tyrosine phosphorylation and COX-2 expression but enhanced dedifferentiation, whereas inhibition of ERK with PD98059 did not affect caveolin-1 tyrosine phosphorylation levels, suggesting that ERK at least is not related to dedifferentiation and COX-2 expression through caveolin-1 tyrosine phosphorylation. **Conclusion:** Our results indicate that SNP in articular chondrocytes stimulates dedifferentiation and inflammatory response via p38 kinase signaling in association with caveolin-1 phosphorylation. **(Immune Network 2006;6(3):117-122)**

Key Words: SNP, caveolin-1, dedifferentiation, inflammatory response, p38 kinase

Introduction

Chondrocytes are differentiated from mesenchymal cells during embryo development (1,2). The phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix (ECM) molecules, including type II collagen and proteoglycans (3-5). Dedifferentiation is a major restriction in mass cell production for cell therapy or tissue engineering of destructive cartilage. Also, dedifferentiation of chondrocytes involves a gradual shift from the synthesis of type II to types I and III collagen. Nitric oxide (NO) produced via inducible NO synthase in articular chondrocytes plays a central role in cartilage diseases such as osteoarthritis and rheumatoid arthritis. NO causes cartilage destruction by inducing apoptosis, dedifferentiation, and inflammatory responses such as cyclooxygenase (COX)-2 expression and prostaglandin E_2 (PGE_2) production in articular chondrocytes (6-9). We showed previously that direct production of NO by treatment with NO donor, sodium nitroprusside (SNP), causes dedifferentiation (10) and COX-2 expression (11) via MAP kinase signaling in rabbit articular chondrocytes. For example, NO-induced activation of extracellular signal-regulated protein kinase (ERK) promotes dedifferentiation, COX-2 expression, and inhibition of apoptosis, whereas NO-induced p38 kinase activation triggers apoptosis, COX-2 expression, and maintains differentiated phenotypes (10,11).

Caveolin, a family of 21～24 kDa membrane pro-

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tein that binds many different sinaling molecules such as heterotrimeric G proteins, Src, Ras and calmodulin (12-16) . Caveolin-1 is also directly involved in signaling cascades as a substrate of both serine and tyrosine kinases. Caveolin was first identified as a major tyrosine-phosphorylated protein in v-Src-transformed embryonic fibroblasts (17) and was demonstrated that tyrosine phosphorylation of caveolin-1 on tyrosine 14 was mediated by the activation of p38 kinase and Src tyrosine kinase (18). Phosphorylation of caveolin-1 on tyrosine is likely to be an intermediate step in a signaling cascade occurring within caveolae.

The present study investigate the roles of caveolin-1 tyrosine phosphorylation in the regulation of dedifferentiation and COX-2 expression of SNP-treated chondrocytes. The investigation additionally focuses on the characterization of the role of MAP kinase subtypes in dedifferentiation and COX-2 expression. We report here that p38 kinase regulates nitric oxide-induced dedifferentiation and COX-2 expression of articular chondrocytes in a caveolin-1 tyrosine phosphorylation-dependent pathway.

Materials and Methods

Monolayer culture of rabbit articular chondrocytes and experimental culture condition. Rabbit articular chondrocytes were released from cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion. To summarize, after aseptic dissection cartilage slices were aseptically dissected and then dissociated enzymatically for 6 h in 0.2% collagenase type II (381 U/mg solid, Sigma) in PBS, and individual cells were then obtained by collecting the supernatant after brief centrifugation. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine-calf serum, 50μg/ml streptomycin, and 50 units/ml penicillin, after which and they were then plated on culture dishes at a density of 5×10^4 cells/cm^2. The medium was changed every 2 days after seeding, and cells reached confluence in approximately 5 days.

Determination of chondrocyte differentiation status. Loss of chondrocyte phenotype, dedifferentiation, was determined by examining the accumulation of sulfated glycosaminoglycan with Alcian Blue, as described previously (19,20) or by the expression of type II collagen studied by immunoblot analysis. To summarize, type II collagen expression was detected using antibodies purchased from Chemicon (Temecula, CA) by immunoblotting, as described below.

Immunoblot analysis. Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecylsulfate, supplemented with protease inhibitors [10μg/ml leupeptin, 10μg/ml pepstatin A, 10μg/ml aprotinin and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride] and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). The proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose sheet was then blocked with 3% non-fat dry milk in Tris-buffered saline. COX-2 was detected using antibody purchased from Cayman Chemical (Ann Arbor, MI). Caveolin-1, p-Caveolin-1, ERK-2 and p38 were detected using antibodies purchased from Santa Cruz Biotech. (Santa Cruz, CA). The bands were visualized using peroxidase-conjugated secondary antibodies and chemiluminescence.

Imunofluorescence microscopy. Tyrosine phosphorylation of caveolin-1 in rabbit articular chondrocytes was investigated using an indirect immunofluorescence microscopy, as described previously (20). Briefly, chondrocytes were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized and blocked with 0.1% Triton X-100 and 5% fetal calf serum in PBS for 30 min. The fixed cells were washed and incubated for 1 h with antibody (10μg/ml) against anti-phospho-caveolin-1 antibodies and with fluorescin-conjugated goat anti-mouse antibodies for 30 min. Cells were then mounted with low-Fade, and observed under a fluorescence microscope.

p38 kinase assay. p38 kinase activity was determined by immune complex kinase assays as described previously (19,20). Briefly, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate and inhibitors of proteases and phosphatases, as described above. Total cell lysates were precipitated with polyclonal anti-p38 kinase antibody and the immune complexes collected using protein-A Sepharose beads. The beads were re-suspended in 20μl kinase reaction buffer containing 25 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$, 5μCi [γ-32P] ATP and 1μg of substrate activating transcription factor-2 (ATF-2) for p38 kinase (New England Biolabs, Beverly, MA). The kinase reaction was performed for 30 min at 30°C and phosphorylated ATF-2 was detected by autoradiography following gel electrophoresis.

PGE_2 assay. PGE_2 production was determined by measuring the levels of cellular and secreted PGE_2 using an assay kit (Amersham Pharmacia Biotech, NJ, UK). Briefly, chondrocytes were seeded in standard 96-well microtiter plates at 2×10^4 cells/well. Following addition of the indicated pharmacological rea-

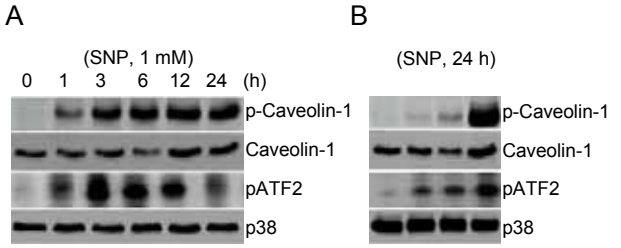

Figure 1. Tyrosine phosphorylation of caveolin-1 and p38 kinase in SNP-treated chondrocytes. A and B, rabbit articular chondrocytes were treated with 1 mM SNP for the indicated time periods (A) or for 24 h with the specified concentrations of SNP (B). Activation of Cav-1 (caveolin-1) was determined by immunoblot analysis, using antibody specific to activated p-Caveolin-1. p38 kinase activity was determined by immunocomplex kinase assay, using ATF-2 as a substrate. Expression of Caveolin-1 and p38 MAP kinase was determined by immunoblot analysis. The data in A represent the results of a typical experiment conducted four times, and B signifies the average values with standard deviation (n=4).

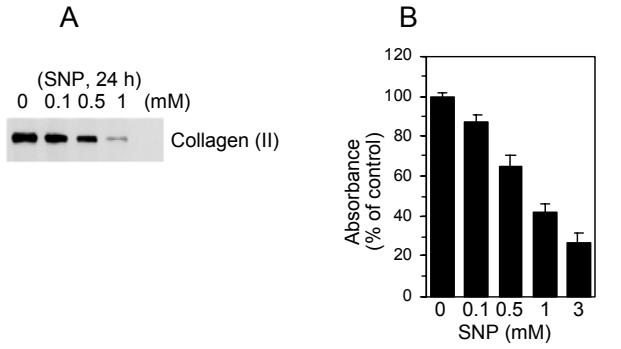

Figure 2. Dedifferentiation in SNP-treated chondrocytes. Chondrocytes were treated with the indicated concentrations of SNP for 24 h. The expression of type II collagen (collagen II) was determined immunoblot analysis (A). Accumulation of sulfated glycosaminoglycan in cells treated with the indicated concentrations of SNP for 24 h was quantified by Alcian Blue staining (B). The data represent the results of a typical experiment conducted at least three times with similar results.

gents, total cell lysate was used to quantify the amount of PGE_2, according to the manufacturer's protocol. PGE_2 levels were calculated against a standard curve of PGE_2 and normalized against the amount of genomic DNA.

Data analyses and statistics. The results are expressed as the means±S.E. values calculated from the specified number of determinations. A Student's test was used to compare individual treatments with their respective control values. A probability of $p < 0.05$ was taken as denoting a significant difference.

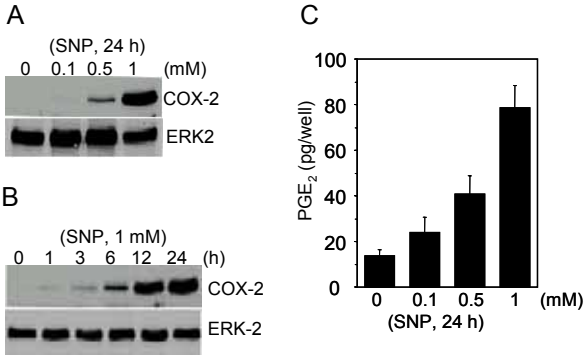

Figure 3. SNP treatment induces COX-2 expression in articular chondrocytes. Chondrocytes were treated with the indicated concentrations of SNP for 24 h (A, C) or with 1 mM SNP for specified time period (B). The expression of COX-2 and ERK-2 was determined immunoblot analysis (A, B), and production of PGE_2 was determined by using a PGE_2 assay kit (C). The data represent the results of a typical experiment conducted at least three times with similar results.

Results

Nitric-oxide-induces phosphorylation of caveolin-1 and p38 kinase. Rabbit articular chondrocytes in primary culture were treated with the NO donor SNP. SNP caused a dramatic increased caveolin-1 tyrosine phosphorylation and p38 kinase activity as determined by immunoblotting and immunocomplex kinase assay, respectively, in a dose- and time-dependent manner, as shown in Fig. 1A and Fig. 1B. But expression of caveolin-1 slightly increased. These results indicate that NO induces caveolin-1 tyrosine phosphorylation and p38 kinase activation.

NO induces dedifferentiation, COX-2 expression, and PGE_2 production of articular chondrocytes. To examine the effects of SNP on articular cartilage chondrocyte on dedifferentiation, COX-2 expression, and PGE_2 production, primary cell cultures were treated with the indicated concentrations of SNP for 24 h or with 1 mM SNP for specified time periods. As expected, expression of type II collagen (Fig. 2A) and sulfated proteoglycan synthesis (Fig. 2B) were significantly reduced in a dose-dependent manner in SNP-treated chondrocytes. But, SNP increased protein levels of COX-2 in a time-and dose-dependent manner as determined by immunoblotting (Fig. 3A,B). Consistent with the induction of COX-2 expression, SNP stimulated PGE_2 production (Fig. 3C). These data indicate that NO not only causes dedifferentiation of articular chondrocytes but also stimulates COX-2 expression and PGE_2 production.

NO-induced activation of p38 kinase causes tyrosine phosphorylation of caveolin-1. To determine the association between caveolin-1 and p38 kinase activation, chondrocytes were treated with SNP in the presence and ab-

sence of SB203580, an inhibitor of p38 kinase and expression and tyrosine phosphorylation of caveolin-1 were examined. The addition of SB203580 to SNP-treated chondrocytes completely blocked the increased tyrosine phosphorylation of caveolin-1 in a dose dependent manner (Fig. 4A). But, caveolin-1 expression is slightly decreased by SB203580. In contrast to the effects of p38 kinase inhibition, blockade of ERK with PD98059, an inhibitor of ERK-1/-2 upstream kinase and did not affect tyrosine phosphorylation and expression of caveolin-1 (Fig. 4B). In an attempt to elucidate the mechanism of p38 kinase regulated caveolin-1 tyrosine phosphorylation, chondrocytes were treated with SNP in the absence or presence of SB203580. SNP treatment resulted in strong staining of caveolin-1 tyrosine phosphorylation, and SB203580 abolished the effects of SNP (Fig. 5). This phenomenon is consistent with immunoblotting. These results indicate that NO-induced caveolin-1 tyrosine phosphorylation is regulated by p38 kinase activity, but not ERK-1/-2 activity.

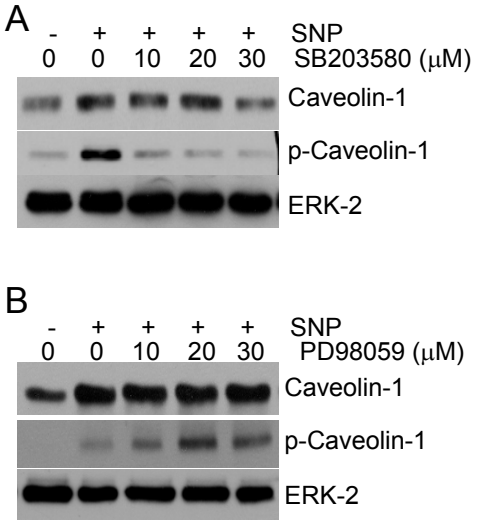

Figure 4. NO-induced activation of p38 kinase but not ERK-1/-2 causes tyrosine phosphorylation of caveolin-1. A and B, cells were treated with 1 mM SNP for 24 h in the presence of indicated concentrations of SB203580 (A) or PD98059 (B). Caveolin, p-Caveolin-1 and ERK-2 were detected by immunoblot analyses. The data represent the average values with standard deviation (n=4).

p38 kinase regulates NO-induced dedifferentiation, COX-2 expression and PGE_2 production. A recent study demonstrated that SNP treatment of articular chondrocytes causes both dedifferentiation (10) and inflammatory response (11). To investigate the expression levels of type II collagen and COX-2, chondrocytes were treated with SNP in the presence and absence of SB203580. Consistent with our previous data (10,11), blockade of p38 kinase accelerated NO-induced decrease of type II collagen expression and COX-2 expression in a dose-dependent manner (Fig. 6A). Similar to the effects on the above results, inhibition of p38 kinase potentiated NO-induced decrease in the accumulation of sulfated proteoglycan (Fig. 6B) and PGE_2 production (Fig. 6C). Taken together, these results indicate that NO-induced dedifferentiation, COX-2 expression and PGE_2 production is regulated through p38 kinase pathway.

Discussion

Our previously studies indicated that NO caused apoptosis and dedifferentiation, which are mediated by MAP kinase subtypes ERK and p38 kinase (10). These MAP kinase play opposing roles, with activated ERK-1/-2 inducing dedifferentiation and inhibiting NO-induced apoptosis, while p38 kinase signaling maintains the differentiated status and induces apoptosis. We also previously demonstrated that NO-stimulates expression of inflammatory molecules such as COX-2 and PGE_2 in chondrocytes via ERK and p38 kinase (11,21).

Caveolin-1 is directly involved in signaling cascades as a substrate of both serine and tyrosine kinases. Caveolin-1 is phosphorylated on tyrosine 14 by Src,

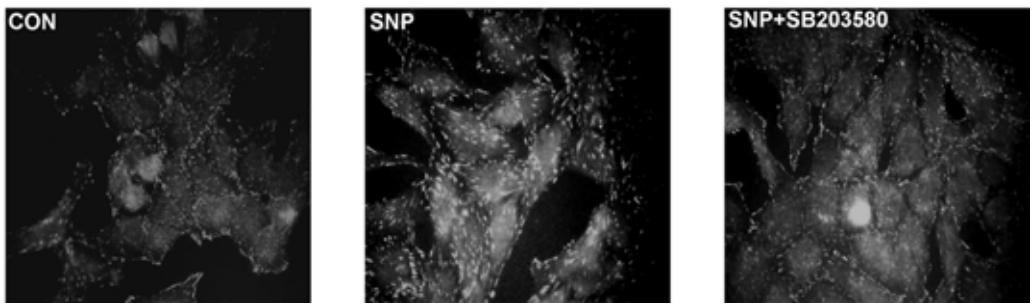

Figure 5. NO-induced tyrosine phosphorylation of caveolin-1. Chondrocytes were untreated or treated with 20μM SB203580 and exposed to 1 mM SNP for 24 h. Tyrosine phosphorylated caveolin-1 (p-Caveolin-1) was immunostained, and photographs were taken with an immunofluorescence microscope. The data represent the results of a typical experiment conducted at least three times.

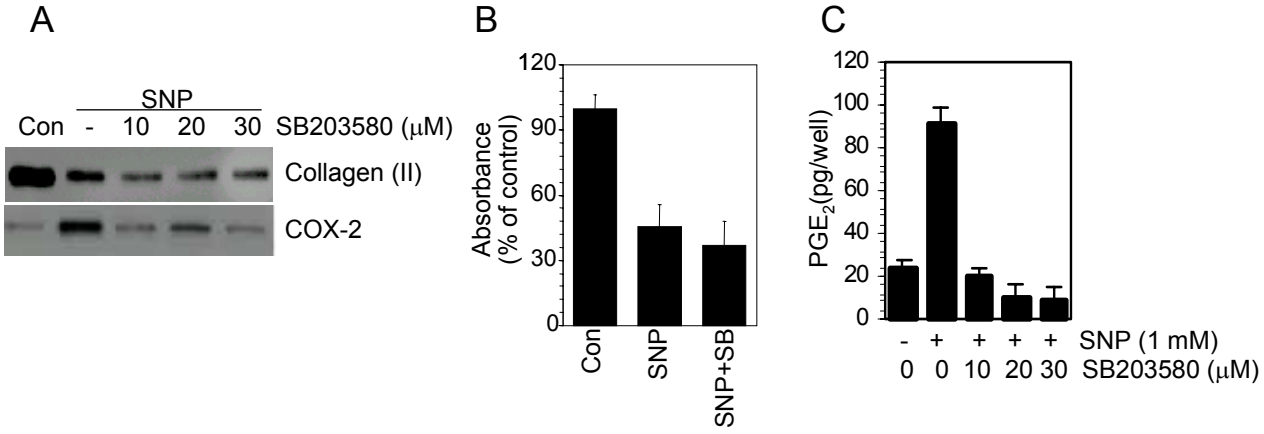

Figure 6. p38 kinase regulates dedifferentiation and COX-2 expression/PGE_2 production. A and C, articular chondrocytes were treated with 1 mM SNP for 24 h in the absence or presence of indicated concentrations of SB203580. Type II collagen (Collagen II) and COX-2 were detected by immunoblotting. Chondrocytes were untreated or treated with 20μM SB203580 and exposed to 1 mM SNP for 24 h. Accumulation of sulfated glycosaminoglycan was quantified by Alcian blue staining (B). Production of PGE_2 was determined by using a PGE_2 assay kit. The data represent the results of a typical experiment conducted at least three times.

Fyn and Abl in response to a number of stimuli, including insulin, angiotensin II, osmotic shock, and oxidative stress (22-24). Numerous studies have demonstrated that caveolin interacts with a number of signaling molecules that are thought to be enriched in caveolae, including certain small and heterotrimeric G proteins (13,25), endothelial nitic oxide synthase (26,27) and a subset of tyrosine kinase receptors (28). In fact, caveolin-1 is a preferred substrate for these tyrosine kinases in cells (29-32).

We first demonstrated signaling pathways involved in NO-induced dedifferentiation, COX-2 expression, and PGE_2 production in rabbit articular chondrocytes, focusing on the functional relationship between caveolin-1 and p38 kinase. Based on results obtained in this study and our previous reports (10,21,33), we propose the following pathway: NO activation of p38 kinase activates caveolin-1 tyrosine phosphorylation. This activation of caveolin-1 is necessary for NO-induced dedifferentiation, COX-2 expression and PGE_2 production. In this regard, p38 kinase have been proposed to interact with caveolin. This possibility will be the subject of future studies.

Footnotes

1. Abbreviations used are: SNP, sodium nitroprusside; NO, nitric oxide; ATF, activating transcription factor; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; PGE_2, prostaglandin E_2.

2. This work was supported by Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2005-202-100654), Basic Research Program of the Korea Science and Engineering Foundation Grant (KOSEF Grant R01-2003-000-10154-0), Rheumatoid Research Center Program (KOSEF Grant R11-2002-098-07003-0).

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