Actin cytoskeletal architecture regulates nitric oxide-induced apoptosis, dedifferentiation and cyclooxygenase-2 expression in articular chondrocytes via mitogen-activated protein kinase and protein kinase C pathways

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Running Title: Actin cytoskeleton in articular chondrocytes

Keywords: Cytochalasin D, F-actin, Type II collagen, Prostaglandin E₂, Signal transduction

Footnotes

Abbreviations

Supported by the National Research Laboratory Program M1-0104-00-0064 (Korea Ministry of Science and Technology) and Rheumatoid Research Center Program R11-2002-098-04005-0 (Korea Science and Engineering Foundation).

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ABSTRACT

Nitric oxide (NO) in articular chondrocytes regulates differentiation, survival and inflammatory responses by modulating extracellular signal-regulated protein kinase (ERK)-1 and -2, p38 kinase and protein kinase C (PKC) $\alpha$ and $\zeta$. In this study, we investigated the effects of the actin cytoskeletal architecture on NO-induced dedifferentiation, apoptosis, cyclooxygenase (COX)-2 expression and prostaglandin E$_2$ (PGE$_2$) production in articular chondrocytes, with a focus on ERK-1/-2, p38 kinase and PKC signaling. Disruption of the actin cytoskeleton by cytochalasin D (CD) inhibited NO-induced apoptosis, dedifferentiation, COX-2 expression and PGE$_2$ production in chondrocytes cultured on plastic or during cartilage explants culture. CD treatment did not affect ERK-1/-2 activation, but blocked the signaling events necessary for NO-induced dedifferentiation, apoptosis, and COX-2 expression such as activation of p38 kinase and inhibition of PKC$\alpha$ and $\zeta$. CD also suppressed activation of downstream signaling of p38 kinase and PKC, such as NF-$\kappa$B activation, p53 accumulation, and caspase-3 activation, which are necessary for NO-induced apoptosis. NO production in articular chondrocytes caused down regulation of phosphatidylinositol (PI) 3-kinase and Akt activities. The down regulation of PI-3 kinase and Akt was blocked by CD treatment, and the CD effects on apoptosis, p38 kinase, and PKC$\alpha$ and $\zeta$ were abolished by the inhibition of PI-3 kinase with LY294002. Our results collectively indicate that the actin cytoskeleton mediates NO-induced regulatory effects in chondrocytes by modulating down regulation of PI-3 kinase and Akt, activation of p38 kinase, and inhibition of PKC$\alpha$ and $\zeta$. 
INTRODUCTION

Chondrocytes are differentiated from mesenchymal cells during embryo development (1, 2). The phenotypes of differentiated chondrocytes in articular cartilage are characterized by the synthesis and maintenance of cartilage-specific extracellular matrix molecules, including type II collagen and proteoglycans such as aggrecan. However, the differentiated phenotype is unstable both in vivo and in vitro, and thus lost by a process designated 'dedifferentiation' upon exposure of cells to interleukin-1β (3, 4), nitric oxide (NO) (5) or retinoic acid (6, 7) and during serial monolayer culture (8, 9). NO mediates the regulation of chondrocyte phenotype and survival by inducing dedifferentiation and apoptosis (5, 10, 11). We previously showed that direct production of NO by treatment of primary culture articular chondrocytes with a NO donor, sodium nitroprusside (SNP), led to apoptosis, dedifferentiation and cyclooxygenase (COX-2) expression via a complex protein kinase signaling cascade involving mitogen-activated protein (MAP) kinase and protein kinase C (PKC) (12-15). 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 (12, 15). NO additionally inhibits protein kinase C (PKC) α and ζ activities (13). PKCα activity is inhibited due to blockage of expression independent of MAP kinase signaling, whereas PKCζ activity is suppressed as a result of p38 kinase activation that follows proteolytic cleavage by caspase-3. Inhibition of PKCα and ζ is necessary for NO-induced dedifferentiation and nuclear factor (NF)-κB activation (15). Activated NF-κB has dual functions, specifically, induction of COX-2 expression and subsequent PGE2 production, and apoptosis by stimulating p53 transcription. A previous study by our group also reveals that p38 kinase stimulates NO-induced apoptosis via p53 accumulation as a result of stabilization due to serine-15 phosphorylation (14).

The actin cytoskeletal architecture is believed to be an important modulator of chondrocyte phenotype. Dedifferentiation of chondrocytes by retinoic acid or serial monolayer culture is accompanied by significant
changes in actin cytoskeletal architecture. Disruption of the actin cytoskeleton by dihydrocytochalasin B promotes redifferentiation of chondrocytes (16, 17). The actin cytoskeleton additionally mediates changes in articular chondrocyte phenotype induced by bone morphogenetic protein (18) or NO (19). Furthermore, disruption of the actin cytoskeleton by cytochalasin D (CD) triggers chondrogenesis in limb mesenchymal cells (20-23), indicating a function in the regulation of chondrogenic differentiation of mesenchymal cells and maintenance of differentiated chondrocyte phenotype. However, to date, the molecular mechanism underlying the regulation of chondrocyte phenotype by actin cytoskeletal architecture has yet to be fully elucidated.

In addition to regulating chondrocyte phenotype, the actin cytoskeleton modulates apoptosis either positively or negatively in a variety of cell types, depending on the experimental system (24-26). For instance, disruption of the actin cytoskeleton causes apoptosis of capillary endothelial cells (27), airway epithelial cells (28), T cells (29), megakaryoblastic leukemia cells (30) and dentate granule cells (31), but inhibits apoptosis in B lymphocytes (32) and lymphoma cells (33). However, it is currently unknown whether actin cytoskeleton also regulates apoptosis of articular chondrocytes.

In view of the evident significance of the actin cytoskeletal architecture in the physiology of chondrocytes and cartilage, we initially investigated its role in NO-induced dedifferentiation, apoptosis and inflammatory responses, such as cyclooxygenase (COX)-2 and prostaglandin E₂ (PGE₂) production in primary culture rabbit articular chondrocytes. We additionally characterized the molecular mechanism of regulation of chondrocyte function by the actin cytoskeleton, focusing on the roles of MAP kinase and PKC. Here we report that disruption of the actin cytoskeleton by CD results in the inhibition of NO-induced dedifferentiation, apoptosis, COX-2 expression and PGE₂ production in articular chondrocytes via modulation of MAP kinase activation and inhibition of PKCα and ζ signaling.
EXPERIMENTAL PROCEDURES

Culture of articular chondrocytes - Articular chondrocytes were isolated from knee joint cartilage slices of two-week-old New Zealand white rabbits by enzymatic digestion with collagenase type II in Dulbecco’s modified Eagle’s medium (DMEM) (9). Cells were maintained in DMEM supplemented with 10% fetal bovine calf serum, 50 µg/ml streptomycin, and 50 units/ml penicillin by plating on culture dishes at a density of 5 × 10⁴ cells/cm² with medium replacement every 2 days. At 3.5-days in culture, cells were treated with the specified pharmacological reagents for 1 h prior to SNP treatment, including CD to disrupt the actin cytoskeleton, jasplakinolide to stabilize the actin cytoskeleton, PD98059 (Calbiochem, La Jolla, CA) to inhibit MEK-1/-2 (34), SB203580 (Calbiochem, La Jolla, CA) to inhibit p38 kinase (35), z-Asp-Glu-Val-Asp-fluoromethyl ketone (z-DEVD; Bachem, Heidelberg, Germany) to inhibit caspase-3 (36), and SN-50 peptide (Biomol, Plymouth Meeting, PA) to inhibit nuclear translocation of NF-κB (37). Where indicated, chondrocytes at day 3 in culture were infected with either control adenovirus or adenovirus coding for wild-type rabbit PKCα or mouse PKCζ inserted into a cosmid cassette. Infected cells were cultured in complete medium for 24 h and treated with 1 mM SNP for an additional 24 h.

Cartilage explants culture - Cartilage slices (~125 mm³) were obtained from rabbit knee joints and maintained in DMEM in the absence or presence of various pharmacological reagents specified in individual experiments. Cartilage explants were fixed in 4% paraformaldehyde for 24 h at 4°C, dehydrated with graded ethanol, embedded in paraffin and sectioned into 4 µm slices. Sections were stained with Alcian blue to detect accumulation of sulfated proteoglycan using standard procedures as described previously (38). Apoptotic cells were determined using the method described below.

Immunofluorescence microscopy - Immunofluorescence microscopy was used to determine F-actin
organization in primary culture articular chondrocytes or cartilage explants, which were either left untreated or treated with various pharmacological reagents. Briefly, primary culture chondrocytes were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized and blocked in PBS containing 0.1% Triton X-100 and 5% fetal calf serum for 30 min. Fixed cells were washed with PBS and incubated for 1 h with rhodamine-conjugated phalloidin, re-washed, and then observed under a standard fluorescence microscope (38).

**Determination of cell death** – NO induces apoptosis in chondrocytes, as previously demonstrated by DNA fragmentation and terminal deoxynucleotidyl transfer-mediated nick end labeling (TUNEL) assays (12). Apoptotic cell death in cartilage explants was determined by TUNEL method using an assay kit (Roche Molecular Biochemicals, Indianapolis, IL). Apoptotic cells were additionally quantified using approximately $1 \times 10^4$ cells on a FACSort flow cytometer with the Cellquest analysis program (Becton Dickinson, Mountain View, CA), as described previously (12).

**Assay of caspase-3 activity** - Caspase-3 activation in SNP-treated chondrocytes was determined by measuring the absorbance of a cleaved synthetic substrate of caspase-3, Ac-Asp-Glu-Val-Asp-chromophore p-nitroaniline (12). Briefly, chondrocytes were lysed on ice for 10 min in cell lysis buffer provided in the CLONTECH A ApoAlert™ CPP32 colorimetric assay kit. Lysates were reacted with 50 μM Ac-Asp-Glu-Val-Asp-chromophore p-nitroaniline in reaction buffer (0.1 M HEPES, 20 % glycerol, 10 mM DTT and protease inhibitors, pH 7.4). Mixtures were maintained at 37 °C for 1 h in a water bath and subsequently analyzed in an ELISA reader. Enzyme activity was calculated from a standard curve prepared using p-nitroaniline. The relative levels of pNA were normalized against the protein concentration of each extract.
**NF-κB reporter gene assay** - NF-κB activation was examined indirectly by analyzing the degradation of inhibitor protein κB (I-κB) using Western blot analysis, and directly with a reporter gene assay. For a reporter gene assay, chondrocytes were transfected with a plasmid containing luciferase and three tandem repeats of serum response element or a control vector, using LipofectAMINE PLUS (12). Transfected cells were cultured in complete medium for 24 h, either left untreated or treated with various pharmacological reagents, and used to determine luciferase activity with an assay kit from Promega (Madison, WI). Luciferase activity was normalized against β-galactosidase activity.

**Immunoprecipitation and kinase assays** - To determine the activities of PKCα, PKCζ and p38 kinase, cell lysates were prepared in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, protease inhibitors [10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 1 μM 4-(2-aminoethyl) benzenesulfonyl fluoride] and phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4). Cell lysates were precipitated with polyclonal antibody against p38 kinase, PKCζ (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or PKCα (BD Transduction Laboratories, Lexington, KY). Immune complexes were collected using protein A Sepharose beads and the kinase reaction was performed in 20 μl reaction buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM MgCl2,[γ-32P] ATP, and 1 μg substrate [activating transcription factor-2 (ATF-2)] for p38 kinase or myelin basic protein for PKCα and ζ. Substrate phosphorylation was detected by autoradiography (13).

**PGE2 assay** - PGE2 production in articular chondrocytes was determined by measuring the levels of cellular and secreted PGE2 with an assay kit purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK), as reported previously (39). Briefly, chondrocytes were seeded in standard 96-well microtiter plates, and
treated with various pharmacological reagents. Total cell lysates were used to quantify the amount of PGE$_2$ according to the manufacturer’s protocol. PGE$_2$ levels were calculated against a standard curve.

*Western blot 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% SDS supplemented with protease inhibitors and phosphatase inhibitors, as described above. Proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Proteins were detected using the following antibodies: anti-type II collagen from Chemicon (Temecula, CA), polyclonal anti-p53 and phosphorylation-specific antibody for ERK from New England Biolabs (Beverly, MA), monoclonal PKC$\alpha$, PKC$\zeta$, and ERK-1/-2 from BD Transduction Laboratories, polyclonal p38 kinase from Santa Cruz Biotechnology Inc. Blots were developed using a peroxidase-conjugated secondary antibody and visualized with an ECL system.

**RESULTS**

*Disruption of the actin cytoskeleton inhibits NO-induced apoptosis of articular chondrocytes* - We have previously shown that direct production of NO by SNP in primary culture articular chondrocytes results in apoptosis and dedifferentiation (12-15). Prior to determining the role of the actin cytoskeleton in NO-induced apoptosis, we examined the organization of actin filaments in articular chondrocytes. The actin cytoskeleton in primary culture chondrocytes at passage 0 comprised a stress fiber-like structure or thick struts of actin fiber extended across the length of the chondrocytes (Fig. 1A). NO induced a typical peripheral distribution of actin filaments that is consistent with their mechanical supporting function for shrinking apoptotic cells. Treatment with CD led to collapse of the filamentous actin structure to amorphous clots of depolymerized protein and a
more round shape of cells (Fig. 1A). The effects of actin cytoskeleton disruption on NO-induced apoptosis were determined by staining cell nuclei with propidium iodide and TUNEL assay. As shown in Fig. 1B, NO induced apoptotic cell death, and disruption of the actin cytoskeleton by CD significantly reduced the number of TUNEL-positive cells. Flow cytometric analyses revealed that CD inhibits NO-induced apoptosis in a dose-dependent manner (Fig. 1C). The role of the actin cytoskeleton in NO-induced apoptosis was further investigated using jasplakinolide, which is a potent inducer of actin polymerization and selectively lock actin in a polymeric state. Treatment with jasplakinolide led to the formation of relatively fine stress fiber and large aggregations of actin filaments (Fig. 2A). Jasplakinolide also increased the number of cells that show NO-induced peripheral distribution of actin filaments (Fig. 2A). Indeed, jasplakinolide dramatically enhanced NO-induced apoptosis in a dose-dependent manner (Fig. 2B). Taken together, the above results clearly indicate that disruption of actin filaments reduces NO-induced apoptosis, whereas stabilization of actin cytoskeleton enhances apoptotic cell death.

In the next series of experiments, we investigated the molecular mechanism of apoptosis regulation by the actin cytoskeleton. Since NO induces chondrocyte apoptosis via NF-κB-dependent accumulation of p53 that in turn leads to caspase-3 activation (12, 13, 15), the effects of actin cytoskeleton disruption on the activation of caspase-3 and NF-κB, and on the accumulation of p53 were determined. CD treatment blocked NO-induced activation of caspase-3 (Fig. 3A), NF-κB [as demonstrated by a NF-κB reporter gene assay and inhibition of IκB degradation (Figs. 3B and 3C)], and accumulation of pro-apoptotic p53 protein (Fig. 3C) in a dose-dependent manner. Since treatment with SN-50 (a NF-κB inhibitor) and zDEVD peptide (a caspase-3 inhibitor) blocked NO-induced apoptosis (Fig. 3D), the above results indicate that inhibition of these activities by disruption of the actin cytoskeleton is responsible for the suppression of cell death.

We previously (13) demonstrated that protein levels and activities of PKCα and ζ, signaling molecules upstream of NF-κB activation and p53 accumulation, decrease upon NO production. Moreover, these events are
necessary for apoptosis and dedifferentiation of chondrocytes. We additionally showed that PKCζ activity is inhibited as a result of p38 kinase activation, whereas suppression of PKCα activity is independent of MAP kinase signaling (12, 13). Accordingly, we examined whether disruption of the actin cytoskeleton blocks NO-induced apoptosis via the modulation of MAP kinase and PKCα and ζ signaling. As depicted in Fig. 4A, expression and activity of PKCα and ζ decreased in SNP-treated chondrocytes. Disruption of the actin cytoskeleton abolished the observed decrease in PKCα and ζ expression and activity. In addition, ectopic expression of PKCα or ζ by adenovirus infection suppressed NO-induced apoptosis (Fig. 4B), indicating that the inhibitory effects of CD on NO-induced apoptosis are a result of blockage of inhibition of PKCα and ζ activity. As expected, SNP treatment in articular chondrocytes also activated ERK-1/2 and p38 kinase, as determined by Western blotting and kinase assays, respectively (Fig. 4C). Disruption of the actin cytoskeleton did not significantly affect NO-induced ERK-1/2 activation (Fig. 4C, upper panel), but activation of p38 kinase by NO was completely blocked (Fig. 4C, lower panel). Treatment with the ERK-1/2 inhibitor, PD98059 (20 µM), potentiated apoptosis, whereas 20 µM SB203580 (a p38 kinase inhibitor) blocked apoptosis (Fig. 3D). Therefore, suppression of p38 kinase activation appears to be responsible for apoptosis inhibition upon actin cytoskeleton disruption.

We recently observed that NO production in articular chondrocytes inhibits cell survival signaling, including phosphatidylinositol 3-kinase (PI3-kinase) and Akt, a process that is necessary for NO-induced apoptosis (40). Based on this finding, we investigated whether CD treatment modulates the cell survival signal to block NO-induced apoptosis. As shown in Fig. 5A (upper panel), NO induced a decrease in both phosphorylated (and thus activated) and total Akt protein levels. NO-induced inhibition of Akt activity and expression was blocked by the disruption of the actin cytoskeleton by CD (Fig. 5A, middle panel). The effects of CD on Akt were abolished by the inhibition of PI3-kinase with LY2944002, a well-known signaling molecule upstream of Akt (Fig. 5A, lower panel). Inhibition of NO-induced apoptosis by disruption of the actin
Cytoskeleton was abolished when cells were treated with LY294002 (Fig. 5C). In addition, inhibition of PI3-kinase and Akt signaling pathway with LY294002 blocked the inhibition of NO-induced apoptotic signaling such as activation of p38 kinase, inhibition of PKCα and ζ, activation of NF-κB, and accumulation of p53 (Fig. 5B). The above results collectively suggest that NO-induced inhibition of PI3-kinase and Akt pathways is required for the activation of apoptotic signaling and, moreover, actin cytoskeleton disruption inhibits apoptosis by stimulating PI3-kinase and Akt survival.

**Disruption of the actin cytoskeleton inhibits NO-induced dedifferentiation of articular chondrocytes** — Next we examined the role of the actin cytoskeleton in the regulation of chondrocyte dedifferentiation. NO-induced loss of the differentiated phenotype of articular chondrocytes, as demonstrated by the reduction of sulfated proteoglycan accumulation and type II collagen expression. CD treatment blocked the NO-induced decrease in sulfated proteoglycan accumulation (Fig. 6A) and expression of type II collagen (Fig. 6C, upper panel) in a dose-dependent manner. In contrast to the effects of CD, stabilization of actin filaments with jasplakinolide potentiated the NO-induced suppression of type II collagen expression (Fig. 6C, lower panel). In addition, disruption of the actin cytoskeleton was accompanied by suppression of chondrocyte dedifferentiation (i.e., decrease in proteoglycan accumulation and type II collagen expression) by interleukin-1β, epidermal growth factor, retinoic acid, and phorbol ester (Figs. 6B and 6C). The above results indicate that the actin cytoskeleton regulates not only NO-induced apoptosis, but also dedifferentiation of primary culture articular chondrocytes.

**IGF-1 inhibits dedifferentiation and apoptosis by modulating actin cytoskeletal architecture** — We recently found that insulin-like growth factor-1 (IGF-1) inhibits NO-induced apoptosis and dedifferentiation of primary culture articular chondrocytes (40). Because the inhibitory effects of CD on NO-induced apoptosis and
dedifferentiation were similar to those of IGF-1, we investigated whether IGF-1 exerts its effects by modulating actin filaments. Similar to the effects of CD, IGF-1 treatment caused disruption of stress fiber and formation of amorphous clots of depolymerized actin (Fig. 7A). The effects of IGF-1 on actin cytoskeleton were abolished by the treatment of jasplakinolide, which causes formation of more stress fiber and aggregations of actin filaments (Fig. 7A). Jasplakinolide abolished IGF-1-induced enhancement of type II collagen expression (Fig. 7B) and completely blocked the inhibitory effects of IGF-1 on NO-induced apoptosis (Fig. 7C). Therefore, the above results suggest that the stimulatory effect and the inhibitor effect of IGF-1 on type II collagen expression and on NO-induced apoptosis, respectively, is due to its ability to disrupt the actin cytoskeleton.

*Actin cytoskeleton regulates COX-2 expression and PGE$_2$ production in articular chondrocytes*—The finding that NO induces COX-2 expression and PGE$_2$ production in articular chondrocytes (15) prompted an investigation into the role of the actin cytoskeleton in the regulation of COX-2 expression and PGE$_2$ production. As expected, NO production caused a significant increase in COX-2 expression and PGE$_2$ production, which was blocked by the disruption of actin filaments with CD treatment (Figs. 8A upper panel and 8B). In contrast, stabilization of actin filaments by jasplakinolide caused COX-2 expression in the absence of NO production and even enhanced NO-induced COX-2 expression (Fig. 8A), indicating a critical role of actin dynamics in NO-induced COX-2 expression and PGE$_2$ production. We focused on the roles of MAP kinase subtypes in regulation by the actin cytoskeleton, since ERK-1/-2 and p38 kinase modulate COX-2 expression and subsequent PGE$_2$ production (41-44). NO triggered the activation of ERK-1/-2 and p38 kinase, as established by Western blot analyses and an *in vitro* kinase assay, respectively (Fig. 8C, upper panel). PD98059, an inhibitor of ERK-1/-2, partially blocked COX-2 expression (Fig. 8B, middle panel) but completely suppressed PGE$_2$ production (Fig. 8C), probably by inhibiting its activity. In contrast, the p38 kinase inhibitor, SB203580, completely abolished both COX-2 expression (Fig. 8B, lower panel) and PGE$_2$ production (Fig. 8C). The results
indicate that activities of ERK-1/-2 and p38 kinase are required for COX-2 expression and subsequent PGE₂ production induced by NO. Since disruption of the actin cytoskeleton blocks NO-induced activation of p38 kinase, but not ERK-1/-2, the above results imply that the inhibition of p38 kinase activation by CD is responsible for the blockage of COX-2 expression and subsequent PGE₂ production.

**Actin cytoskeleton regulates NO-induced apoptosis, dedifferentiation and COX-2 expression in articular chondrocytes during cartilage explants culture** - Since the response of chondrocytes in monolayer culture may differ from that in a three-dimensional natural matrix, we examined whether the cytoskeletal architecture in chondrocytes regulates NO-induced apoptosis, dedifferentiation and COX-2 expression during cartilage explants culture. F-actin was strongly stained in the peripheral region of chondrocytes in cartilage explants. SNP treatment resulted in strong homogenous staining of F-actin, and CD reversed the effects of NO production (Fig. 9A). Analogous to the data obtained with chondrocytes cultured on plastic, NO induced an increase in TUNEL-positive chondrocytes and reduced Alcian blue staining. The effects of NO were blocked upon treatment of cartilage explants with CD (Fig. 9A). CD additionally inhibited NO-induced COX-2 expression in chondrocytes during cartilage explants culture (Fig. 9B). Thus, disruption of the actin cytoskeletal architecture blocks NO-induced apoptosis, dedifferentiation and COX-2 expression in chondrocytes cultured either on plastic or a three-dimensional natural matrix.

**DISCUSSION**

CD is a fungal metabolite that inhibits actin polymerization and disrupts existing actin filaments by binding to high-affinity sites located at their polymerization ends. Jasplakinolide, which is a macrocyclic peptide isolated from the marine sponge *Jaspis johnstoni*, induces actin polymerization and stabilizes actin in a polymeric state. Using CD and jasplakinolide, we show that the actin cytoskeletal architecture is a critical
regulator of articular chondrocyte function, including differentiation, survival and inflammatory responses, such as COX-2 expression and PGE$_2$ production, both in primary culture on plastic or in a natural matrix (i.e., cartilage explants). The physiological significance of the actin dynamics in differentiation and apoptosis was demonstrated by the observation that IGF-1 inhibits NO-induced apoptosis and dedifferentiation by modulating actin cytoskeletal architecture. We additionally demonstrate that the actin cytoskeleton regulates these functions by modulating PI3-kinase, Akt, MAP kinases, and PKC$\alpha$ and $\zeta$ signaling. The actin cytoskeleton not only provides cells with mechanical stability, but also serves as a major scaffold to organize parts of the signaling machinery that regulates adhesion and intercellular communications. The cytoskeletal architecture undergoes rapid and dramatic changes in conformation and arrangement in response to cell stimulation, which in turn possibly transduce signals that result from surface-receptor occupation, ultimately leading to cell activation and proliferation. The ability of the actin cytoskeleton to organize the cytoplasm by compartmentalizing and localizing proteins means that it can act as a scaffolding element for signaling intermediates. Indeed, enzymatic activities of actin-associated proteins (such as casein kinase II) are modified upon binding directly to actin (45).

To date, the mechanisms underlying the regulation of signaling molecules, including PI3-kinase, Akt, p38 kinase and PKC, by the actin cytoskeleton are not clearly understood. Disruption of the actin cytoskeleton did not affect ERK activation, but blocked activation of p38 kinase and inhibition of PKC$\alpha$ and $\zeta$. Since PKC$\zeta$ activity is blocked as a result of p38 kinase activation (13), it is likely that blockage of PKC$\zeta$ inhibition by CD is due to suppression of the upstream signaling molecule, p38 kinase. The signaling molecule mediating NO-induced inhibition of PKC$\alpha$ that is independent of MAP kinase signaling remains to be identified. We did not attempt to determine the target molecules regulated by CD to modulate PKC$\alpha$ in this study. However, our results indicate that p38 kinase-dependent and -independent PKC$\zeta$ and $\alpha$ regulate NF-$\kappa$B activation that leads to the accumulation of p53 and activation of caspase-3, and consequently chondrocyte apoptosis (15). Therefore, inhibition of NO-induced apoptosis by disruption of the actin cytoskeleton is consistent with the suppression of
the apoptotic signaling pathway, such as activation of p38 kinase, inhibition of PKCα and ζ, NF-κB activation, p53 accumulation, and caspase-3 activation. Our data additionally indicate that disruption of the actin cytoskeleton blocks NO-induced inhibition of survival signals such as PI3-kinase and Akt. Moreover, inhibition of the PI3-kinase and Akt pathways is required for the blockage of p38 kinase activation, inhibition of PKCα and ζ, NF-κB activation, accumulation of p53, and caspase-3 activation, although the molecular mechanisms of action are not elucidated in this study. Actin is a known substrate of caspases (46, 47), and is cleaved in a caspase-3-dependent manner during NO-induced apoptosis (data not shown). It is suggested that actin depolymerization is linked to DNA degradation (46) and either inhibits (27-31) or promotes apoptosis (32, 33), depending on the cell type.

The observation that the actin cytoskeleton regulates chondrocyte phenotype is consistent with earlier reports. For example, chondrocytes lose their differentiated phenotype during in vitro culture, a process that is blocked by disruption of the actin cytoskeleton (16, 17, 48). The re-expression of differentiated chondrocyte phenotype upon actin cytoskeleton disruption is independent of cell shape change, but mediated by reorganization of the actin cytoskeletal architecture (17). However, the molecular mechanism mediating the maintenance of differentiated phenotype is yet to be elucidated. Consequently, we investigated the role of the actin cytoskeleton in NO-induced dedifferentiation. Our data show that disruption of the actin structure induces suppression of dedifferentiation by blocking NO-induced inhibition of PKCα and ζ. This conclusion is consistent with our previous observation that ectopic expression of PKCα and ζ blocks NO-induced dedifferentiation (13). Although inhibition of p38 kinase activation by CD is consistent with blockage of the downstream signaling molecule PKCζ, it is not critical for the inhibition of NO-induced dedifferentiation. This conclusion is based on the finding that inhibition of NO-induced p38 kinase acts as a signal to potentiate NO-induced dedifferentiation (12). We therefore speculate that blockage of the inhibition of PKCα and ζ, rather than the inhibition of p38 kinase activation, as a result of actin cytoskeleton disruption is responsible for the
suppression of dedifferentiation. Our current results also indicated that actin dynamics regulates dedifferentiation of chondrocytes caused by interleukin-1β, epidermal growth factor, retinoic acid and phorbol ester. It is well established that IL-1β produces NO in chondrocytes via iNOS expression, and the inhibition of iNOS with LNMMA blocks IL-1β-induced dedifferentiation of chondrocytes. We also confirmed that LNMMA inhibits NO production and dedifferentiation (data not shown). Contrast to IL-1β, PMA or EGF did not cause NO production, and hence LNMMA did not affect PMA- or EGF-induced dedifferentiation (data not shown). Our current observation that disruption of actin filaments blocks dedifferentiation caused not only by NO production (such as by IL-1 beta and SNP) but also by PMA and EGF, therefore, indicates that actin filaments are common target for the regulation of dedifferentiation by various dedifferentiating agonists.

Our current investigation clearly shows that actin cytoskeleton disruption blocks COX-2 induction and PGE2 production induced by NO. We demonstrate that actin cytoskeleton disruption abrogates COX-2 induction and PGE2 production, due to its ability to block activation of p38 kinase. Our data are consistent with reports by other groups that p38 kinase regulates COX-2 expression and subsequent PGE2 production in various cell types (40-44). In agreement with our results in chondrocytes, disruption of microtubules led to an increase in COX-2 expression and PGE2 release mediated by p38 kinase in human mammary epithelial cells (49), and actin cytoskeleton disruption triggered COX-2 expression and PGE2 release in human umbilical endothelial cells (50). Although PGE2 is known to regulate differentiation of chondrocytes (51, 52), have previously shown that indomethacin inhibits NO-induced dedifferentiation and apoptosis independent of the expression of COX2 and PGE2 production. Furthermore, specific inhibition of COX-2 with NS398 that completely blocked NO-induced PGE2 production, but did not affect apoptosis and accumulation of sulfated proteoglycans. In addition, treatment of chondrocytes with various concentrations of PGE2 in the absence or presence of SNP did not affect cell viability, accumulation of sulfated proteoglycan, and or type II collagen expression (53). Therefore, it is likely that COX-2 expression and PGE2 production is not a cause of NO-induced dedifferentiation and apoptosis.
ACKNOWLEDGEMENTS

We thank Dr. Sunghoe Chang for his helpful discussions and Seon-Hee Kim for her technical assistance in immunohistochemistry.

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Neuropharmacology 42, 1109-1118


FIGURE LEGENDS

Figure 1. Disruption of the actin cytoskeleton inhibits NO-induced apoptosis of primary culture articular chondrocytes. A and B. Articular chondrocytes were left untreated (Control) or treated with 1 mM SNP without (SNP) or with 0.5 µM CD (CD+SNP) for 24 h. Cells were stained for F-actin with rhodamine-conjugated phalloidin (A). Nuclear staining with propidium iodide (PI) and TUNEL assay were performed to detect apoptotic cells (B). C. Articular chondrocytes were left untreated (Control) or treated with the indicated concentrations of CD and 1 mM SNP for 24 h. Apoptosis was determined by flow cytometry. Data are presented as results of a typical experiment (A and B) and as mean values with standard deviation (B) (n = 4).

Figure 2. Stabilization of actin filaments with jasplakinolide potentiates NO-induced apoptosis. A, Articular chondrocytes were treated for 24 h with DMSO as a control, 50 nM jasplakinolide, 1 mM SNP without (SNP) or with 50 nM jasplakinolide. Cells were stained for F-actin with rhodamine-conjugated phalloidin. B, Articular chondrocytes were left untreated (Control) or treated with the indicated concentrations of jasplakinolide and 1 mM SNP for 24 h. Apoptosis was determined by flow cytometry. Data are presented as results of a typical experiment (A) and as mean values with standard deviation (B) (n = 4).

Figure 3. Inhibition of NO-induced activation of caspase-3 and NF-κB, and accumulation of p53 upon disruption of the actin cytoskeleton. A-C, Articular chondrocytes were left untreated (Control) or treated with the indicated concentrations of CD and 1 mM SNP for 24 h. Activities of caspase-3 (A) and NF-κB (B) were determined using the protocol described in Experimental Procedures. Protein levels of I-κB and p53 were determined by Western blotting (C). D. Articular chondrocytes were left untreated (Control) or treated for 24 h with 1 mM SNP without (SNP) or with 50 µg/ml SN-50 peptide (SN50+SNP) or 20 µM zDEVD peptide (DEVD+SNP). Apoptosis was determined by flow cytometry. Data are presented as mean values with standard deviation.
deviation (A, B, C) and as results of a typical experiment (D). At least 4 independent experiments were performed.

**Figure 4. Disruption of actin cytoskeleton modulates NO-induced PKCα and ζ and MAP kinase signaling.**

**A,** Articular chondrocytes were left untreated (Control) or treated with the indicated concentrations of CD and 1 mM SNP for 24 h. Protein levels and activities of PKCα and ζ were determined by Western blot analysis and *in vitro* kinase assay, respectively. **B,** Articular chondrocytes were infected with control adenovirus or adenovirus containing PKCα or PKCζ, and cultured in complete medium for 24 h. Cells were left untreated (Control) or treated with 1 mM SNP for 24 h. Apoptosis was determined by flow cytometry. **C,** Chondrocytes were left untreated (Control) or treated with 1 mM SNP with the indicated concentrations of CD for 24 h. Levels of pERK-1/-2, ERK-2 and p38 kinase were determined by Western blot analysis, whereas p38 kinase activity was determined using the immunocomplex kinase assay with ATF-2 as a substrate. **D,** Articular chondrocytes were left untreated (Control) or treated with 1 mM SNP for 24 h in the absence or presence of 20 µM PD98059 or SB202190. Apoptotic cells were determined by flow cytometry. Data are presented as a typical result (A and C) and mean values with standard deviation (B and D). At least four independent experiments were performed.

**Figure 5. Actin cytoskeleton modulates apoptotic signaling via PI3-kinase and Akt signaling pathways.**

Chondrocytes were treated with 1 mM SNP for the indicated time-periods (upper panel), untreated (Control) or treated for 24 h with 1 mM SNP and the indicated concentrations of CD (middle panel), and either left untreated or treated with 1 mM SNP in the absence or presence of 0.5 µM CD and the specified concentrations of LY294002 (lower panel). Levels of total and phosphorylated Akt were determined by Western blot analysis. **B** and **C,** Chondrocytes were treated with 1 mM SNP in the absence or presence of 0.5 µM CD and the indicated concentrations of LY294002. Levels of phosphorylated ERK, PKCα and ζ, IκB, and p53 were determined by
Western blot analysis, and activities of p38 kinase and PKC α and ζ were determined using the immunocomplex kinase assay (B). Apoptotic cells were quantified by flow cytometry (C). Data are presented as a typical result (A and B) out of four independent experiments or mean values with standard deviation (C).

**Figure 6. Inhibition of articular chondrocyte dedifferentiation upon disruption of the actin cytoskeleton.**

Chondrocytes were left untreated or treated with 1 mM SNP and the indicated concentrations of CD for 24 h. Accumulation of sulfated proteoglycan (A) and expression of type II collagen (C, upper panel) was determined by Alcian blue staining and Western blot analysis, respectively. Chondrocytes were left untreated or treated with 5 ng/ml interleukin (IL)-1β, 5 ng/ml epidermal growth factor (EGF), 100 nM retinoic acid (RA) or 100 nM phorbol 12-myristate 13-acetate (PMA) with or without 0.5 µM CD for 72 h. Accumulation of sulfated proteoglycan (B) was determined by Alcian blue staining. Type II collagen expression (C, middle panel) was determined by Western blot analysis. The cells were treated with the indicated concentrations of jasplakinolide in the absence and presence of 1 mM SNP for 24 h. Expression of type II collagen was determined by Western blotting. Data are presented as mean values with standard deviation or as results of a typical experiment. At least 4 independent experiments were performed.

**Figure 7. IGF-1 regulates type II collagen expression and apoptosis by modulating actin cytoskeletal architecture.**

A and B, Articular chondrocytes were treated for 24 h with DMSO as a control, 50 nM jasplakinolide, 100 ng/ml of IGF-1 without or with 50 nM jasplakinolide. Cells were stained for F-actin with rhodamine-conjugated phalloidin (A) and expression of type II collagen and ERK-1 was determined by Western blotting (B). C, Articular chondrocytes were left untreated or treated for 24 h with 1 mM SNP, 100 ng/ml of IGF-1, and the indicated concentrations of jasplakinolide. Apoptosis was determined by flow cytometry. Data are presented as results of a typical experiment (A and B) and as mean values with standard deviation (C) (n = 4).
Figure 8. CD treatment blocks NO-induced COX-2 expression and PGE\textsubscript{2} production by inhibiting p38 kinase. \textbf{A}, Chondrocytes were left untreated (Control) or treated with 1 mM SNP for 24 h with the indicated concentrations of CD (upper panel). Alternatively, the cells were treated for 24 h with the indicated concentrations of jasplakinolide in the absence or presence of 1 mM SNP (lower panel). COX-2 expression was determined by Western blotting. \textbf{B}, Chondrocytes were left untreated (Control) or treated with 1 mM SNP for 24 h with 0.5 \textmu M CD. PGE\textsubscript{2} levels were determined as described in Experimental Procedures. \textbf{C}, Chondrocytes were treated with 1 mM SNP for the indicated time-periods, and phosphorylation of ERK-1/-2 (pERK) and p38 kinase (pATF-2) was determined by Western blot analysis and \textit{in vitro} kinase assays, respectively (upper panel). Chondrocytes were left untreated (-) or treated (+) with 1 mM SNP in the presence of the indicated concentrations of PD98059 (PD) or SB203580 (SB). COX-2 expression and ERK-1/-2 phosphorylation were determined by Western blot analysis. p38 kinase activity and COX-2 expression were analyzed by Western blotting and \textit{in vitro} kinase assay, respectively (lower panel). \textbf{D}, Chondrocytes were left untreated (Control) or treated with 1 mM SNP in the absence or presence of 10 \textmu M PD98059 (PD) or SB203580 (SB). The amount of PGE\textsubscript{2} produced was determined. Data are presented as mean values with standard deviation or as results of a typical experiment out of four independent experiments.

Figure 9. Actin cytoskeleton regulates NO-induced apoptosis, dedifferentiation, and COX-2 expression in articular chondrocytes during cartilage explants culture. Cartilage explants were left untreated or treated with 1 mM SNP for 72 h in the absence or presence of 1 \textmu M CD. Apoptotic cells were determined using the TUNEL assay, sulfated proteoglycan synthesis by Alcian blue staining, and F-actin organization by staining with rhodamine-conjugated phalloidin (A). COX-2 expression was determined using immunohistochemical analyses (B). Data are presented as results of a typical experiment out of at least four independent experiments.
Fig. 1, Kim et al.

A

Control
SNP
CD+SNP

B

PI
TUNEL

C

Apoptotic cells (% of total)

SNP (1 mM)

CD (µM)
Fig. 2, Kim et al.

A

Control SNP

Apoptotic cells (% of total)

B

Jasplakinolide

SNP (1 mM)

Jasplakinolide

Jasplakinolide+SNP

Apoptotic cells (% of total)

Jasplakinolide (nM)

Control 0 25 50 100 200

SNP (1 mM)
Fig. 3, Kim et al.
Fig. 4, Kim et al.
Fig. 5, Kim et al.
Fig. 7, Kim et al.
Fig. 8, Kim et al.
Fig. 9, Kim et al.

A

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