Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit nitric oxide-induced apoptosis and dedifferentiation of articular chondrocytes independent of cyclooxygenase activity

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Footnotes
1 Abbreviations used are: ATF: activating transcription factor; COX: cyclooxygenase; ERK: extracellular signal-regulated protein kinase; MAP kinase: mitogen-activated protein kinase; NFκB: nuclear factor κB; NO: nitric oxide; NSAIDs: nonsteroidal anti-inflammatory drugs; PG: prostaglandin, PKC: protein kinase C; SNP: sodium nitroprusside.

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ABSTRACT

Nitric oxide (NO) causes apoptosis and dedifferentiation of articular chondrocytes by the modulation of extracellular signal-regulated kinase (ERK), p38 kinase, and protein kinase C (PKC) α and ζ. In this study, we investigated the effects and mechanisms of non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, ketoprofen, ibuprofen, sulindac sulfide, and flurbiprofen in NO-induced apoptosis and dedifferentiation of articular chondrocytes. We found that all of the examined NSAIDs inhibited apoptosis and dedifferentiation. NO production in chondrocytes caused activation of ERK-1/-2 and p38 kinase, both of which oppositely regulate apoptosis and dedifferentiation. NO production also caused inhibition of PKCα and ζ independent of and dependent on p38 kinase, respectively, that is required for apoptosis and dedifferentiation. Among the signaling molecules modulated by NO, NSAIDs blocked NO-induced activation of p38 kinase, potentiated ERK activation, and blocked inhibition of PKCα and ζ. NSAIDs also inhibited some of the apoptotic signaling that is downstream of p38 kinase and PKC, such as NFκB activation, p53 accumulation, and caspase-3 activation. The inhibitory effects of NSAIDs on apoptosis and dedifferentiation were independent of the inhibition of cyclooxygenase (COX)-2 and prostaglandin E₂ (PGE₂) production, as evidenced by the observation that specific inhibition of COX-2 activity and PGE₂ production or exogenous PGE₂ did not affect NO-induced apoptosis and dedifferentiation. Taken together, our results indicate that NSAIDs block NO-induced apoptosis and dedifferentiation of articular chondrocytes by the modulation of ERK, p38 kinase, and PKCα and ζ in a manner independent of their ability to inhibit COX-2 and PGE₂ production.
INTRODUCTION

Chondrocytes are a unique cell type in which the differentiated phenotype is reversible. The phenotype of chondrocytes is regulated by the balance between anabolic and catabolic reactions of molecules, which are involved in the maintenance of cartilage homeostasis (1). Differentiated chondrocytes both in vivo and in vitro dedifferentiate into fibroblastic cells upon exposure to interleukin-1β (2, 3), retinoic acid (4), or nitric oxide (NO) (5). Although the molecular mechanism is not yet clear, dedifferentiation of articular chondrocytes is believed to play a role in the pathophysiology of arthritis. In addition to dedifferentiation, increased apoptotic death of chondrocytes was observed in arthritic cartilage and apoptosis is closely related to cartilage destruction (6, 7), indicating that chondrocyte apoptosis plays an important role in the pathogenesis of arthritis.

NO is generally believed to be an important mediator of the dedifferentiation and apoptosis of articular chondrocytes in arthritic cartilage (5, 8, 9). NO is produced in chondrocytes by the action of pro-inflammatory cytokines such as interleukin-1β. NO production in chondrocytes causes activation of matrix metalloproteinases (10), decreased production of interleukin-1 receptor antagonists (11), inhibition of proteoglycan synthesis and type II collagen expression (12, 13), and apoptosis of chondrocytes (6, 7, 14). Indeed, inhibition of NO production protects against damage of cartilage and chondrocytes in a number of experimental models. For instance, in experimentally induced osteoarthritis in a range of animal species, a significant correlation was observed between the level of NO and the prevalence of apoptotic cells in cartilage tissue (7). Moreover, inhibition of NO resulted in reduced articular cartilage damage and apoptotic cell death (11, 15). In our previous studies, we have shown that direct production of NO by treating chondrocytes with an NO donor, sodium nitroprusside (SNP), induces apoptosis and dedifferentiation of primary culture articular chondrocytes (16-18). NO-induced apoptosis and dedifferentiation of articular chondrocytes were regulated by opposite functions of mitogen-activated protein (MAP) kinase subtypes, extracellular signal-regulated protein kinase (ERK) and p38 kinase (16). NO-induced activation of ERK-1/-2 induces dedifferentiation with the inhibitory effects on
apoptosis, whereas activation of p38 kinase induces apoptosis and is responsible for the maintenance of differentiated phenotype. In addition to MAP kinase signaling, NO production caused the inhibition of protein kinase C (PKC) α and ζ activities (17). The inhibition of PKCα activity is due to inhibition of its expression, which is independent of MAP kinase signaling. In contrast, PKCζ activity is blocked as a result of p38 kinase activation, and inhibition of PKCζ activity is followed by proteolytic cleavage by caspase-3. We also found that p38 kinase induces NO-induced apoptosis by accumulating p53 via NFκB-dependent transcription and stabilization by serine 15 phosphorylation (18).

NSAIDs such as aspirin and indomethacin have been used to relieve pain and inflammation in arthritic cartilage. NSAIDs exert their effects primarily by the inhibition of cyclooxygenase (COX), a key enzyme that converts arachidonic acid to prostaglandin (PG) (19). In addition to the alleviation of inflammation, some NSAIDs also modulate homeostasis of chondrocyte and cartilage such as matrix molecule synthesis. For example, several NSAIDs such as sodium salicylate inhibit proteoglycan synthesis, but others such as nimesulide induce cartilage matrix synthesis, while others including piroxicam have no effect on matrix synthesis (20, 21). In addition, some NSAIDs such as nimesulide and ibuprofen have a protective effect in staurosporine-induced apoptosis of chondrocytes (22). Several lines of evidence suggest that some of the effects of NSAIDs are independent of the inhibition of COX (23). Indeed, it has been shown that NSAIDs modulate COX-independent signaling pathways such as Ras (24), NFκB (25), AP-1 (26), ERK (26), p38 kinase (27), and others. Because the role of NSAIDs in the maintenance of homeostasis and apoptosis of articular chondrocytes is not clearly understood, although the action of NSAIDs in inflammation is clear, we investigated the function of various NSAIDs in NO-induced dedifferentiation and apoptosis and characterized the molecular mechanism of NSAIDs action in articular chondrocytes.

EXPERIMENTAL PROCEDURES

Isolation of rabbit articular chondrocytes and culture conditions - Articular chondrocytes were isolated
from knee joint cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion as described previously (28). Briefly, cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 units/mg solid, Sigma) in Dulbecco’s modified Eagle’s medium. After collecting individual cells by brief centrifugation, the cells were resuspended in culture medium supplemented with 10% (v/v) fetal bovine calf serum, 50 µg/ml streptomycin, and 50 units/ml penicillin, and then plated on culture dishes at a density of $5 \times 10^4$ cells/cm$^2$. The medium was replaced every 2 days after plating, and cells reached confluence at approximately 5 days of culture. Cells from day 4 cultures were treated with various concentrations of the indicated pharmacological reagents for 1 h prior to SNP treatment. These reagents included various NSAIDs, PD98059 (Calbiochem, La Jolla, CA) to inhibit MEK-1/-2 (29), SB203580 (Calbiochem, La Jolla, CA) to inhibit p38 kinase (30), z-Asp-Glu-Val-Asp-fluoromethyl ketone (z-DEVD; Bachem, Heidelberg, Germany) to inhibit caspase-3 (31), or SN50 (Biomol, Plymouth Meeting, PA) to inhibit nuclear translocation of NFκB (32).

In some experiments where indicated, chondrocytes from day 3 cultures were infected with either control adenovirus or adenovirus containing wild type rabbit PKCα or mouse PKCζ that was inserted into a cosmid cassette, pAxCAwt. Infected cells were cultured in complete medium for 24 h and were then treated with 1.5 mM SNP for an additional 24 h.

**Cartilage explants culture** - Rabbit joint cartilage explants (~125 mm$^3$) were cultured in Dulbecco’s modified Eagle’s medium in the absence or presence of various pharmacological reagents as indicated in each experiment. The 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 as described previously (33). The sections were stained by standard procedures using Alcian blue to determine differentiation status of chondrocytes. Apoptotic cells were determined by the procedure described below.
**Determination of caspase-3 activity** - Activation of caspase-3 was determined by measuring the absorbance of the cleaved synthetic substrate of caspase-3, Ac-Asp-Glu-Val-Asp-chromophore-p-nitroaniline, as described previously (16). Briefly, chondrocytes were lysed on ice for 10 min in the cell lysis buffer provided in the CLONTECH A ApoAlert™ CPP32 colorimetric assay kit. The lysates were reacted with 50 µM Ac-Asp-Glu-Val-Asp-chromophore-p-nitroaniline in a reaction buffer (0.1 M Hepes, 20 % glycerol, 10 mM DTT, and protease inhibitors, pH 7.4). The mixtures were maintained at 37 °C for 1 h in a water bath and subsequently analyzed in an ELISA reader. The 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.

**Determination of apoptosis** - We have previously shown that NO-induced death of primary culture of articular chondrocytes is due to apoptosis, as demonstrated by DNA fragmentation and terminal deoxynucleotidyl transfer-mediated nick end labeling (TUNEL) (16). In this study, apoptotic cells were quantified by counting surviving cells using an MTT assay kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. Apoptosis of articular chondrocytes in cartilage explants culture was determined by TUNEL assay according to the manufacturer’s protocol (Roche Molecular Biochemicals).

**Immunoprecipitation and kinase assays** - The activity of PKCα, PKCζ, and p38 kinase was determined by an immune complex kinase assay. Briefly, 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 mM 4-(2-aminoethyl) benzenesulfonyl fluoride] and phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4). The cell lysates were precipitated with polyclonal antibodies against p38 kinase or PKCζ (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or PKCα (BD Transduction Laboratories, Lexington, KY). Immune
complexes were collected by protein A Sepharose beads, and resuspended in 20 µl of kinase 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 of substrate [activating transcription factor-2 (ATF-2)] for p38 kinase or myelin basic protein for PKCα and ζ. The phosphorylation of substrates was detected by autoradiography.

**NFκB luciferase assay** - NFκB activity was determined by a reporter gene assay. Briefly, chondrocytes were transfected with plasmid containing luciferase and three tandem repeats of serum response element or a control vector. Transfection of the expression vector was performed by using LipofectAMINE PLUS as described previously (18). The transfected cells were cultured in complete medium for 24 h and used to determine luciferase activity using an assay kit from Promega (Madison, WI). Luciferase activity was normalized against β-galactosidase activity.

**PGE₂ assay** - PGE₂ production in articular chondrocytes was determined by measuring the levels of cellular and secreted PGE₂ by using a PGE₂ assay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Briefly, chondrocytes were plated in standard 96-well microtitre plates at a density of 2 × 10⁴ cells/well. Following treatment with various pharmacological reagents as indicated in each experiment, the total cell lysate was used to quantify the amount of PGE₂ according to the manufacturer’s protocol. PGE₂ levels were calculated against a standard curve of PGE₂.

**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. The proteins were size-fractionated by SDS-
polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Proteins were detected using the following antibodies: type II collagen from Chemicon (Temecula, CA); rabbit anti-p53 polyclonal antibody and phosphorylation-specific antibody for ERK from New England Biolabs (Beverly, MA); PKCα, PKCζ, and ERK-1/-2 from BD Transduction Laboratories; p38 kinase from Santa Cruz Biotechnology Inc. Blots were developed using a peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system.

RESULTS

NSAIDs block NO-induced apoptosis of primary culture articular chondrocytes - Direct production of NO by SNP treatment causes dedifferentiation and apoptosis of articular chondrocytes (16-18). To examine the effects of NSAIDs on articular chondrocyte function, we first examined the effects of NSAIDs on NO-induced apoptotic cell death. Consistent with our previous reports, SNP treatment in rabbit articular chondrocytes caused apoptosis in a dose-dependent manner that was demonstrated by the reduction of cell viability determined by MMT assay (Fig. 1A). The reduction of cell viability was detectable as early as 3 h after SNP treatment (data not shown). The effects of NSAIDs on the survival of articular chondrocytes were examined by pretreating chondrocytes with various concentrations of NSAIDs for 1 h prior to exposure to 1.5 mM SNP for 18 h. As shown in Fig. 1B, all of the examined NSAIDs including indomethacin, ketoprofen, ibuprofen, sulindac sulfide, and flurbiprofen blocked chondrocyte apoptosis in a dose-dependent manner. These results indicate that NSAIDs block NO-induced apoptosis of articular chondrocytes.

NSAIDs block NO-induced dedifferentiation of primary culture articular chondrocytes - We also examined the effects of NSAIDs on NO-induced dedifferentiation of articular chondrocytes. NO production caused loss of the differentiated phenotype of articular chondrocytes as demonstrated by the reduction of sulfated proteoglycan accumulation and type II collagen expression (16). Pretreatment of chondrocytes with
indomethacin blocked the NO-induced decrease in sulfated proteoglycan accumulation as determined by Alcian blue staining (Fig. 2A, upper panel) and type II collagen expression as determined by Western blot analysis (Fig. 2A, lower panel). Treatment of chondrocytes with indomethacin alone did not affect the accumulation of sulfated proteoglycan or type II collagen expression (Fig. 2A). Similar to the effects of indomethacin, other NSAIDs examined in this study (i.e., ketoprofen, ibuprofen, sulindac sulfide, and flurbiprofen) also blocked the NO-induced decrease in proteoglycan accumulation and type II collagen expression in a dose-dependent manner (Fig. 2B). These results indicate that NSAIDs inhibit not only NO-induced apoptosis but also dedifferentiation of primary culture articular chondrocytes.

**NSAIDs block NO-induced apoptosis and dedifferentiation of articular chondrocytes during cartilage explants culture** - Because it is possible that the responses of chondrocytes in monolayer culture may differ from their responses in a 3-dimensional natural matrix, we examined whether NO production causes apoptosis and dedifferentiation of chondrocytes during cartilage explants culture and whether NSAIDs also block NO-induced apoptosis and dedifferentiation. Similar to the effects on chondrocytes cultured on plastic, NO production caused apoptotic cell death and inhibition of sulfated proteoglycan synthesis in chondrocyte in cartilage explants as demonstrated by TUNEL assay and Alcian blue staining, respectively (Fig. 3). In addition, pretreatment of chondrocytes with indomethacin blocked the NO-induced apoptosis and decrease in sulfated proteoglycan accumulation (Fig. 3), indicating that indomethacin blocks NO-induced apoptosis and dedifferentiation of chondrocytes cultured either on plastic or in a 3-dimensional natural matrix.

**Modulation of NO-induced activation of ERK and p38 kinase by indomethacin** - We next investigated the molecular mechanism of NSAIDs modulation of apoptosis and dedifferentiation. We used indomethacin as a NSAID and first examined the possible modulation of NO-induced activation of the MAP kinase subtypes,
ERK-1/-2 and p38 kinase, because these MAP kinase subtypes oppositely regulate NO-induced apoptosis and dedifferentiation (16). SNP treatment caused transient activation of ERK-1/-2 and p38 kinase as determined by Western blot analysis and an in vitro kinase assay, respectively (Fig. 4A, upper panel). Treatment of chondrocytes with indomethacin prior to SNP treatment slightly but consistently enhanced NO-induced ERK-1/-2 activation (Fig. 4A, middle panel). In contrast, NO-induced activation of p38 kinase was blocked by indomethacin treatment in a dose-dependent manner (Fig. 4A, lower panel). Inhibition of NO-induced ERK-1/-2 activation with 20 µM PD98059 resulted in the potentiation of apoptosis and a blockade of dedifferentiation, whereas inhibition of p38 kinase with 20 µM SB203580 blocked apoptosis with the potentiation of dedifferentiation (Fig. 4B and C). The present results are consistent with our previous observation (16) and indicate that NO-induced ERK activation causes dedifferentiation and inhibits apoptosis, whereas activation of p38 kinase induces apoptosis and inhibits dedifferentiation. Therefore, the inhibitory effects of indomethacin on NO-induced apoptosis and dedifferentiation with the potentiation and inhibition of ERK and p38 kinase, respectively, suggest that inhibition of apoptosis by indomethacin is due to blockade of p38 kinase and potentiation of ERK. In contrast, the above results also suggest that modulation of MAP kinase subtypes by indomethacin is not responsible for the inhibition of NO-induced dedifferentiation because the potentiation of ERK and inhibition of p38 kinase is a condition that enhances dedifferentiation.

**Indomethacin blocks NO-induced inhibition of PKCα and ζ activity** - A previous study from our lab (17) indicated that inhibition of PKCα and ζ activities is required for NO-induced apoptosis and dedifferentiation of articular chondrocytes. We also demonstrated that inhibition of PKCζ is due to activation of p38 kinase, whereas inhibition of PKCα is independent of MAP kinase signaling (17). We therefore examined whether indomethacin blocks NO-induced dedifferentiation and apoptosis by modulating PKCα and ζ signaling. Consistent with our previous results (17), expression and activity of PKCα and ζ decreased over time in SNP-
treated chondrocytes (Fig. 5A). The NO-induced decrease in expression and activity of PKCα and ζ was completely blocked by the pretreatment of indomethacin in a dose-dependent manner (Fig. 5B). Moreover, ectopic expression of PKC α or ζ by adenovirus infection blocked NO-induced dedifferentiation (Figs. 5C and 5D) and apoptosis (Fig. 5E). Therefore, the above results suggest that the inhibitory effects of indomethacin on NO-induced dedifferentiation are due to the blockade of NO-induced inhibition of PKCα and ζ activity, whereas the inhibition of apoptosis by indomethacin is due to its ability to modulate both MAP kinase subtypes and PKCα and ζ. Consistent with the inhibition of apoptosis, indomethacin also blocked the signaling molecules downstream of p38 kinase during NO-induced apoptosis such as the activation of NFκB that was demonstrated by the inhibition of IκB degradation (Fig. 6A) and NFκB reporter gene assay (Fig. 6B), the accumulation of p53 (Fig. 6A), and the activation of caspases-3 (Fig. 6C).

**NSAIDs inhibit apoptosis and dedifferentiation independent of the expression of COX-2 and production of PGE2** - Because NSAIDs block COX-2 activity and PGE2 production and because PGE2 is known to regulate differentiation of chondrocytes (34, 35), we examined whether the inhibition of apoptosis and dedifferentiation by NSAIDs is due to their ability to block COX-2 activity and PGE2 production. NO-induced COX-2 expression (Fig. 7A, upper panel) and PGE2 production (Fig. 7B) were blocked by all of the examined NSAIDs, and the effects of indomethacin on COX-2 expression were dose-dependent (Fig. 7A, lower panel). Although a low concentration of indomethacin (i.e., 70 µM) did not significantly block COX-2 expression (Fig. 7A), it completely blocked PGE2 production (Fig. 7C), indicating that COX-2 was blocked at this concentration. Interestingly, although PGE2 production was completely blocked at 70 µM indomethacin, the effects on apoptosis and proteoglycan synthesis were observed at higher concentrations of indomethacin (Fig. 7C), suggesting that the ability of indomethacin to modulate apoptosis and dedifferentiation is independent of the inhibition of COX-2 and PGE2 production.
This possibility was further examined by the specific inhibition of COX-2 with NS398. As shown in Fig. 8A, NS398 (5 µM) 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 (Fig. 8B), or type II collagen expression (Fig. 8D, upper panel). The COX-2 and PGE₂-independent regulation of apoptosis and dedifferentiation were further demonstrated by inhibiting ERK-1/2 and p38 kinase that block NO-induced COX-2 expression (Fig. 8D, lower panel) and PGE₂ production (Fig. 8C). Although inhibition of both MAP kinase subtypes blocked PGE₂ production, NO-induced apoptosis and dedifferentiation were oppositely regulated by ERK-1/2 and p38 kinase (Fig. 8C). Taken together, these results indicate that the ability of indomethacin to block NO-induced apoptosis and dedifferentiation is independent of the inhibition of COX-2 activity and PGE₂ production.

**DISCUSSION**

NO production in articular chondrocytes plays a central role in the pathophysiology of arthritis (5, 8, 9, 36). High levels of nitrite/nitrate are found in the synovial fluid and serum of arthritis patients (37), and it has been shown that NO causes loss of a differentiated phenotype and apoptosis of articular chondrocytes (6, 7, 12-14). We have previously shown that direct production of NO causes apoptosis and dedifferentiation of articular chondrocytes by the activation of ERK-1/2 and p38 kinase and inhibition of PKCα and ζ (16-18). In this study, we investigated the effects of various NSAIDs on NO-induced apoptosis and dedifferentiation of articular chondrocytes cultured on plastic or in a 3-dimensional natural matrix (i.e., explants culture), and found that all of the examined NSAIDs inhibit apoptosis and dedifferentiation in both conditions independent of the inhibition of COX-2 expression and PGE₂ production. As summarized in Fig. 9, we also demonstrated that the inhibitory effects of NSAIDs on apoptosis are due to their ability to potentiate NO-induced ERK activation, to inhibit...
activation of p38 kinase, and to block inhibition of PKCα and ζ. In contrast, the inhibition of NO-induced
dedifferentiation by NSAIDs is due to the blockade of PKCα and ζ signaling but not the modulation of ERK-1/2
and p38 kinase signaling.

The cellular effects of NSAIDs are exerted primarily by the inhibition of COX and PGE2 production.
PGE2 is known to regulate differentiation (34, 35) and apoptosis (14, 38) of chondrocytes depending on the
experimental system. Because NO production in chondrocytes causes COX-2 expression and PGE2 production,
we thought a possibility that NSAIDs modulate NO-induced apoptosis and dedifferentiation by inhibiting COX-
2. However, our current results clearly indicate that the inhibitory effects of NSAIDs on NO-induced apoptosis
and dedifferentiation are independent of the inhibition of COX-2 and PGE2 production. This conclusion is
clearly demonstrated by the specific inhibition of COX-2 activity and by the observation that PGE2 production
or exogenous PGE2 did not affect NO-induced apoptosis and dedifferentiation. In addition, the concentration of
indomethacin that is required for the inhibition of NO-induced apoptosis and dedifferentiation is higher than that
needed for the inhibition of PGE2 synthesis. This is consistent with observations that the COX-independent
actions of NSAIDs such as inhibition of cell cycle progression (39, 40), induction of apoptosis (41-43), and
inhibition of angiogenesis (44, 45), require high concentrations of NSAIDs that are 100- to 1000-fold higher
than those needed to inhibit prostaglandin synthesis.

Our results indicated that the inhibition of NO-induced apoptosis of chondrocytes by NSAIDs is related
to the potentiation of ERK activation, blockade of p38 kinase activation, blockade of PKCα and ζ inhibition,
and inhibition of down stream apoptotic signaling including NFκB, p56, and caspase-3 as summarized in Fig.
9. The modulation of MAP kinase subtypes by indomethacin (i.e., inhibition of p38 kinase and potentiation of
ERK) appears to be involved in the inhibition of NO-induced apoptosis based on the observation that NO-
induced activation of p38 kinase induces apoptosis, whereas ERK activation inhibits apoptosis. The inhibition of
p38 kinase and apoptosis by indomethacin is consistent with the inhibition of apoptotic signaling molecules.
located downstream of p38 kinase, such as PKCζ, NFκB, p53, and caspase-3 (Fig. 9). In contrast to the inhibition of apoptosis, the inhibitory effects of indomethacin on NO-induced dedifferentiation is due to its ability to block NO-induced inhibition of PKCα and ζ, but not its ability to modulate MAP kinase subtypes. This conclusion is based on the observations that NO-induced inhibition of PKCα and ζ is required for the induction of dedifferentiation as well as apoptosis and that the potentiation and inhibition of ERK and p38 kinase, respectively, is the signaling events leading to the potentiation of dedifferentiation (Fig. 9). Modulation of ERK and p38 kinase by indomethacin is independent of its ability to block COX activity, consistent with previously reported results. Indeed, many studies have indicated that COX-independent effects of NSAIDs are exerted by the modulation of ERK and p38 kinase. For example, NSAIDs such as aspirin and sodium salicylate exert their effects by the inhibition ERK-1/2 (26, 45-47), whereas p38 kinase is activated by sodium salicylate in human fibroblasts and is associated with induction of apoptosis (27).

Our current results clearly indicate that indomethacin blocked the NO-induced inhibition of PKCα and ζ activity that is required for the induction of apoptosis and dedifferentiation of SNP-treated chondrocytes (17). Because the activity of PKCζ is blocked as a result of p38 kinase activation (17), it is likely that the effects of indomethacin on PKCζ are due to the inhibition of p38 kinase signaling. However, the possibility that indomethacin directly regulates PKCζ activity cannot be ruled out, although no evidence supports the direct action of NSAIDs in the regulation of PKC isoforms. Nevertheless, it is apparent that the blockade of the NO-induced inhibition of PKCζ by indomethacin inhibits both apoptosis and dedifferentiation of SNP-treated chondrocytes. In contrast to PKCζ, the inhibition of PKCα expression and activity is due to the inhibition of its expression independent of ERK and p38 kinase signaling (17). Based on the observation that inhibition of PKCα activity is a prerequisite for the induction of apoptosis and dedifferentiation, the MAP kinase-independent inhibition of PKCα activity by indomethacin is also essential for the inhibitory effects of indomethacin on apoptosis and dedifferentiation. The mechanisms of indomethacin regulation of PKCα
expression and activity remain to be determined although it is possible that indomethacin regulates PKC\(\alpha\) either directly or indirectly by modulating upstream signaling events. Nevertheless, because our present results indicated that indomethacin-induced inhibition of p38 kinase and potentiation of ERK is not directly involved in the inhibition of dedifferentiation as discussed above, we conclude that the blockade of the inhibition of PKC\(\alpha\) and \(\zeta\) activities by indomethacin plays a critical role in the inhibition of NO-induced dedifferentiation.

Consistent with the inhibition of apoptotic signaling mediators such as p38 kinase and PKC, indomethacin also blocked their downstream signaling molecules such as the activation of NF\(\kappa\)B, accumulation of p53, and activation of caspase-3. Indeed, it has been shown that some types of NSAIDs including ibuprofen, sulindac, sulindac sulfide, and flurbiprofen are able to inhibit NF\(\kappa\)B activation, whereas indomethacin, ketoprofen, and ketorolac are ineffective (23). Using a reporter gene assay and I\(\kappa\)B degradation, we found that ectopic expression of wild type PKC\(\alpha\) or \(\zeta\) by adenovirus infection in chondrocytes blocked the NO-induced activation of NF\(\kappa\)B\(^2\), indicating that p38 kinase-dependent and -independent PKC\(\zeta\) and \(\alpha\) regulates NF\(\kappa\)B activation. We also found that NF\(\kappa\)B activation is required for COX-2 expression\(^2\). This suggests that inhibition of PKC\(\alpha\) and \(\zeta\) is an upstream signaling event leading to NF\(\kappa\)B activation that causes COX-2 expression as summarized in Fig. 9. Given the inability of indomethacin to inhibit NF\(\kappa\)B (23), it is highly likely that the inhibitory effects of indomethacin on NF\(\kappa\)B activation observed in this study are due to the inhibition of its upstream signaling molecules, i.e., p38 kinase and PKC, rather than to a direct action on NF\(\kappa\)B.

In summary, we found that various NSAIDs block apoptosis and dedifferentiation of articular chondrocytes caused by NO production in a manner independent of their ability to inhibit COX-2 and PGE\(_2\) production. The inhibitory effects of NSAIDs on apoptosis are derived from their ability to block NO-induced activation of p38 kinase, to potentiate ERK activation, and to block inhibition of PKC\(\alpha\) and \(\zeta\) whereas the inhibition of NO-induced dedifferentiation by NSAIDs is due to the blockade of PKC\(\alpha\) and \(\zeta\) signaling but not the modulation of ERK-1/-2 and p38 kinase signaling. Additionally, the effects of NO production and NSAIDs
treatment on chondrocytes derived from adult rabbit joint cartilage (from 4-month-old rabbits) or human osteoarthritic cartilage obtained from patients undergoing total knee arthroplasty are essentially same as in growth plate chondrocytes (from 2-week-old rabbits) (data not shown). Because NO production via inducible NO synthase in articular chondrocytes plays a central role in the pathophysiology of arthritis by causing inflammation, apoptosis, dedifferentiation, and the activation of matrix metalloproteinases, our results suggest that NSAIDs have protective effects on cartilage damage, not only by alleviating inflammation but also by inhibiting NO-induced apoptosis and dedifferentiation of articular chondrocytes.

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REFERENCES

1. Sandell, L. J., and Aigner, I. (2001) Arthritis Res. 3, 107-113

2. Frazer, A., Bunning, R. A., Thavarajah, M., Seid, J. M., and Russell, R. G. (1994) Osteoarthritis Cartilage 2, 235-245

3. Demoor-Fossard, M., Redini, F., Boittin, M., and Pujol, J. P. (1998) Biochim. Biophys. Acta 1398, 179-191

4. Horton, W. E., Yamada, Y., and Hassell, J. R. (1987) Dev. Biol. 123, 508-516

5. Amin, A. R., and Abramson, S. B. (1998) Curr. Opin. Rheumatol. 10, 263-268

6. Blanco, F. J., Guitian, R., Vazquez-Martul, E., de Toro, F. J., and Galdo, F. (1998) Arthritis Rheum. 41, 284-289
7. Hashimoto, S., Oche, R. L., Komiya, S., and Lotz, M. (1998) *Arthritis Rheum.* 41, 1632-1638

8. Amin, A. R., Attur, M., Abramson, S. B. (1999) *Curr. Opin. Rheumatol.* 11, 202-209

9. Abramson, S. B., Attur, M., Amin, A. R., and Clancy, R. (2001) *Curr. Rheumatol. Rep.* 3, 535-541

10. Tamura, T., Nakanishi, T., Kimura, Y., Hattori, T., Sasaki, K., Norimatsu, H., Takahashi, K., and Takigawa, M. (1996) *Endocrinology.* 137, 3729-3737

11. Jouzeau, J. Y., Pacquelet, S., Boileau, C., Nedelec, E., Presle, N., Netter, P., and Terlain, B. (2002) *Biorheology.* 39, 201-214.

12. Cao, M., Westerhausen-Larson, A., Niyibizi, C., Kavalkovich, K., Georgescu, H. I., Rizzo, C. F., Hebda, P. A., Stefanovic-Racic, M., and Evans, C. H. (1997) *Biochem. J.* 324, 305-310

13. Taskiran, D., Stefanovic-Racic, M., Georgescu, H. I., and Evans, C. H. (1994) *Biochem. Biophys. Res. Commun.* 200, 142-148

14. Notoya, K., Jovanovic, D. V., Reboul, P., Johanne, M. P., Mineau, F., and Pelletier, J. P. (2000) *J. Immunol.* 165, 3402-3410

15. Pelletier, J. P., Jovanovic, D., Fernandes, J. C., Manning, P. T., Connor, J. R., Currie, M. G., Di Battista, J. A., and Martel-Pelletier, J. (1998) *Arthritis Rheum.* 41, 1275-1286

16. Kim, S.-J., Ju, J.-W., Oh, C.-D., Yoon, Y.-M., Song, W.-K., Kim, J.-H., Yoo, Y.-J., Bang, O.-S., Kang, S.-S., and Chun, J.-S. (2002) *J. Biol. Chem.* 277, 1332-1339

17. Kim, S.-J., Kim, H.-G., Oh, C.-D., Hwang, S.-G., Song, W.-K., Yoo, Y.-J., Kang, S.-S., and Chun, J.-S. (2002) *J. Biol. Chem.* 277, 30375-30381

18. Kim, S.-J., Hwang, S.-G., Shin, D.-Y., Kang, S.-S., and Chun, J.-S. (2002) *J. Biol. Chem.* 277, 33501-33508

19. Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S. Van De Putte, L. B., and Lipsky, P. E. (1998) *FASEBJ.* 12, 1063-1073

20. Ding, C. (2002) *Inflammation* 26, 139-142
21. Dingle, J. T. (1999) *Rheumatology*. **58**, 125-129

22. Mukherjee, P., Rachita, C., Aisen, P. S., and Pasinetti, G. M. (2001) *Clin. Exp. Rheumatol*. **19**, S7-11

23. Tegeder, I., Pfleischsifer, J., Geisslinger, G. (2001) *FASEB J*. **15**, 2057-2072

24. Herrmann, C., Block, C., Geisen, C., Haas, K., Weber, C., Winde, G., Moroy, T., and Muller, O. (1998) *Oncogene* **17**, 1769-1776

25. Kopp, E., and Ghosh, S. (1994) *Science*. **265**, 956-959

26. Huang, C., Ma, W. Y., Hanenberger, D., Cleary, M. P., Bowden, G. T., and Dong, Z. (1997) *J. Biol. Chem.* **272**, 26325-26331

27. Schwenger, P., Skolnik, E. Y., and Vilcek, J. (1998) *Mol. Cell. Biol.* **18**, 78-84

28. Yoon, Y.-M., Kim, S.-J., Oh, C.-D., Ju, J.-W., Song, W.-G., Yoo, Y.-J., Huh, T.-L., and Chun, J.-S. (2002) *J. Biol. Chem.* **277**, 8412-8420

29. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270**, 27489-27494

30. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) *FEBS Lett.* **364**, 229-233

31. Varghese, J., Chattopadhaya, S., and Sarin, A. (2001) *J. Immunol.* **166**, 6570-6577

32. Lin, Y. Z., Yao, S. Y., Veach, R. A., Torgerson, T. R., and Hawiger, J. (1995) *J Biol Chem.* **270**, 14255-14258

33. Ryu, J.-H., Kim, S.-J., Kim, S.-H., Oh, C.-D., Hwang, S.-G., Chun, C.-H., Oh, S.-H., Seong, J.-K., Huh, T.-L., and Chun, J.-S. (2002) *Development* **129**, 5541-5550

34. Goldring, M. B., Suen, L. F., Yamin, R., and Lai, W. F. (1996) *Am. J. Ther.* **3**, 9-16

35. Schwartz, Z., Gilley, R. M., Sylvia, V. L., Dean, D. D., and Boyan, B. D. (1998) *Endocrinology* **139**, 1825-1834

36. Martel-Pelletier, J., Alaaeddine, N., and Pelletier, J. P. (1999) *Frontiers Biosci.* **4**, 694-703
37. Farrell, A. J., Blake, D. R., Palmer, R. M., and Moncada, S. (1992) *Ann. Rheum. Dis.* **51**, 1219-1222

38. von Knethen, A. V., and Brune, B. (1997) *FASEB J.* **11**, 887-895

39. Shiff, S. J., Qiao, L., Tsai, L. L., and Rigas, B. (1995) *J. Clin. Invest.* **96**, 491-503

40. Goldberg, Y., Nassif, I. I., Pittas, A., Tsai, L. L., Dynlacht, B. D., Rigas, B., and Shiff, S. J. (1996) *Oncogene* **12**, 893-901

41. Barnes, C. J., Cameron, I. L., Hardman, W. E., and Lee, M. (1998) *Br. J. Cancer* **77**, 573-580

42. Giardina, C., Boulares, H., and Inan, M. S. (1999) *Biochim. Biophys. Acta* **1448**, 425-438

43. Shao, J., Fujiwara, T., Kadowaki, Y., Fukazawa, T., Waku, T., Itoshima, T., Yamatsuji, T., Nishizaki, M., Roth, J. A., and Tanaka, N. (2000) *Oncogene* **19**, 726-736

44. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. (1998) *Cell* **93**, 705-716

45. Jones, M. K., Wang, H., Peskar, B. M., Levin, E., Itani, R. M., Sarfèh, I. J., and Tamawski, A. S. (1999) *Nat. Med.* **5**, 1348-1349

46. Schwenger, P., Skolnik, E. Y., and Vilcek, J. (1996) *J. Biol. Chem.* **271**, 8089-8094

47. Pillinger, M. H., Capodici, C., Rosenthal, P., Kheterpal, N., Hanft, S., Philips, M. R., and Weissmann, G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **24**, 14540-14545
FIGURE LEGENDS

Figure 1. NSAIDs inhibit NO-induced apoptosis of primary culture articular chondrocytes. Articular chondrocytes were treated with 1.5 mM SNP for the indicated time periods (A) or were treated with the indicated concentrations (µM) of various NSAIDs including indomethacin (Indo), ketoprofen (Keto), ibuprofen (Ibup), sulindac sulfide (Suli), and flurbiprofen (Flur) for 1 h and then exposed to 1.5 mM SNP for 18 h (B). Cell viability was determined by an MTT assay. Data are represented as mean values with standard deviation (n = 4).

Figure 2. NSAIDs inhibit NO-induced dedifferentiation of primary culture articular chondrocytes. Articular chondrocytes were treated with the indicated concentrations (µM) of indomethacin (Indo) (A) or ketoprofen (Keto), ibuprofen (Ibup), sulindac sulfide (Suli), and flurbiprofen (Flur) (B) for 1 h and then exposed to 1.5 mM SNP for 18 h. Accumulation of sulfated glycosaminoglycans was determined by Alcian blue staining (upper panels) and expression of type II collagen was determined by Western blot analysis (lower panels). The data represent mean values with standard deviation and results of a typical experiment selected from at least 4 independent experiments.

Figure 3. NSAIDs block NO-induced apoptosis and dedifferentiation of articular chondrocytes during cartilage explants culture. Cartilage explants were untreated or treated with 1.5 mM SNP for 72 h in the absence or presence of 280 µM indomethacin. Apoptotic cells were determined by TUNEL assay and synthesis of sulfated proteoglycan was determined by Alcian blue staining. The data represent results of a typical experiment selected from at least 4 independent experiments.

Figure 4. Indomethacin modulates NO-induced activation of p38 kinase and ERK-1/-2. A, Chondrocytes
were treated with 1.5 mM SNP for the indicated time periods (upper panel), or cells were treated with 1.5 mM SNP for 12 h (middle and lower panels) in the presence of indicated concentrations (µM) of indomethacin (Indo). NO-induced activation of ERK-1/-2 and p38 kinase in SNP-treated chondrocytes was determined by Western blot analysis or a kinase assay respectively. **B-C,** Chondrocytes were treated with 1.5 mM SNP for 18 h in the absence or presence of 20 µM PD98059 or SB202190, and then cell viability, the accumulation of sulfated glycosaminoglycan, and the expression of type II collagen were determined by MTT assay, Alcian blue staining, or Western blot analysis, respectively. The data represent mean values with standard deviation (**B** and **C**) (n = 4) or a typical result (**A**).

**Figure 5.** Indomethacin blocks the NO-induced inhibition of PKCα and ζ activities. **A** and **B,** Articular chondrocytes were treated with 1.5 mM SNP for the indicated time periods (**A**) or were treated with 1.5 mM SNP for 18 h in the presence of the indicated concentration (µM) of indomethacin (Indo) (**B**). Levels of PKCα and ζ proteins were determined by Western blot analysis and the activities of PKCα and ζ proteins were determined by a kinase assay. **C-E,** Chondrocytes were infected with control adenovirus (Control and SNP) or adenovirus containing PKCα or ζ cDNA. Infected cells were cultured in complete medium for 24 h and treated with 1.5 mM SNP for an additional 24 h. Expression of type II collagen (Coll-II), and PKCζ was determined by Western blot analysis (**C**). Accumulation of sulfated glycosaminoglycan was determined by Alcian blue staining (**D**). Cell viability was determined by an MTT assay (**E**). The data shown represent a typical result (**A** - **C**) or the mean value with standard deviation (**D, E**) from at least 4 independent experiments.

**Figure 6.** Inhibition of NO-induced NFκB activation, p53 accumulation, and caspase-3 activation by indomethacin. Articular chondrocytes were treated with 1.5 mM SNP for 18 h in the presence of the indicated concentration (µM) of indomethacin (Indo) (**A**) or 1.5 mM SNP for 18 h in the absence or presence of 50 µg/ml
SN-50 peptide (B) or 20 µM DEVD (C). Degradation of IκB and accumulation of p53 protein were determined by Western blot analysis (A). NFκB activity (B) and caspase-3 activity (C) were determined as described in Experimental Procedures. Data represent results of a typical experiment (A) or mean values with standard deviation (B and C) (n = 4).

Figure 7. Inhibition of NO-induced COX-2 expression and PGE₂ production by NSAIDs. Chondrocytes were untreated (Control) or were treated with 1.5 mM SNP for 18 h in the absence or presence of 1 mM ketoprofen (Keto), 1 mM ibuprofen (Ibup), 0.1 mM sulindac sulfide (Suli), 1 mM flurbiprofen (Flur), or of the indicated concentration (µM) of indomethacin (Indo). Expression of COX-2 protein was determined by Western blot analysis (A) and production of PGE₂ was determined by using a PGE₂ assay kit (B). Cell viability and accumulation of sulfated glycosaminoglycan were determined by an MTT assay or Alcian blue staining, respectively (C). The data in A represent results of a typical experiment conducted five times and the data in B and C represent mean values with standard deviation from at least 4 independent experiments.

Figure 8. Inhibition of NO-induced apoptosis and dedifferentiation by NSAIDs is independent of the blockade of COX-2 expression and PGE₂ production. Articular chondrocytes were treated with 1.5 mM SNP for 18 h in the absence or presence of 5 µM NS398 (A), the indicated concentration (ng/ml) of PGE₂ (B), 20 µM PD98059, 20 µM SB202190, or 200 ng/ml PGE₂ (C and D). The production of PGE₂, cell viability, and the accumulation of sulfated glycosaminoglycan were determined as described in Experimental Procedures (A-C). Expression of type II collagen and COX-2 was determined by Western blot analysis (D). The data in A - C represent mean values with standard deviation; data in D represent results of a typical experiment (n = 4).

Figure 9. Schematic summary of NSAIDs modulation of signaling pathway during NO-induced de-
differentiation and apoptosis of articular chondrocytes. NSAIDs inhibits NO-induced apoptosis by blocking NO-induced activation of p38 kinase, potentiating ERK activation, and blocking inhibition of PKCα and ζ, whereas the inhibition of NO-induced dedifferentiation by NSAIDs is due to the blockade of PKCα and ζ signaling but not the modulation of ERK-1/-2 and p38 kinase. The inhibitory effects of NSAIDs on apoptosis and dedifferentiation are independent of their ability to block COX-2 activity and PGE₂ production.
Fig. 2 Yoon et al.

A

Absorbance (% of control)

Vehicle SNP

Indo

Type II collagen

B

Absorbance (% of control)

Control Keto Ibup Suli Flur

SNP

Type II collagen

Indo

0 70 140 280

-SNP

+SNP

Type II collagen

Control Vehicle Keto Ibup Suli Flur

SNP
Fig. 3 Yoon et al.
Fig. 4 Yoon et al.
Fig. 5  Yoon et al.
Fig. 6  Yoon et al.
Fig. 8  Yoon et al.
Fig. 9  Yoon et al.

NO → p38 → PKCζ → ? → PKCα → Dedifferentiation

- Activated by NSAIDs
- Inhibited by NSAIDs

- Activation
- Inhibition

ERK

NFκB → COX-2/PGE2

p53 → Caspase → Apoptosis
Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit nitric oxide-induced apoptosis and dedifferentiation of articular chondrocytes independent of the regulation of cyclooxygenase activity

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