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The Induction of Cell Death in Human Osteoarthritis Chondrocytes by Nitric Oxide Is Related to the Production of Prostaglandin E_2 Via the Induction of Cyclooxygenase-2

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There is increasing evidence suggesting that chondrocyte death may contribute to the progression of osteoarthritis (OA). This study focused on the characterization of signaling cascade during NO-induced cell death in human OA chondrocytes. The NO generator, sodium nitroprusside (SNP), promoted chondrocyte death in association with DNA fragmentation, caspase-3 activation, and down-regulation of Bcl-2. Both caspase-3 inhibitor Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-CH_2F and caspase-9 inhibitor Z-Leu-Glu(OCH_3)-His-Asp(OCH_3)-CH_2F prevented the chondrocyte death. Blocking the mitogen-activated protein kinase pathway by the mitogen-activated protein kinase kinase 1/2 inhibitor PD98059 or p38 kinase inhibitor SB202190 also inhibited the SNP-mediated cell death, suggesting possible requirements of both extracellular signal-related protein kinase 1/2 and p38 kinase for the NO-induced cell death. Furthermore, the selective inhibition of cyclooxygenase (COX)-2 by NS-398 or the inhibition of COX-1/COX-2 by indomethacin blocked the SNP-induced cell death. The chondrocyte death induced by SNP was associated with an overexpression of COX-2 protein (as determined by Western blotting) and an increase in PGE_2 release. PD98059 and SB202190, but neither Z-DEVD FMK nor Z-LEHD FMK completely inhibited the SNP-mediated PGE_2 production. Analysis of interactions between PGE_2 and the cell death showed that PGE_2 enhanced the SNP-mediated cell death, whereas PGE_2 alone did not induce the chondrocyte death. These data indicate that NO-induced chondrocyte death signaling includes PGE_2 production via COX-2 induction and suggest that both extracellular signal-related protein kinase 1/2 and p38 kinase pathways are upstream signaling of the PGE_2 production. The results also demonstrate that exogenous PGE_2 may sensitize human OA chondrocytes to the cell death induced by NO. *The Journal of Immunology,* **2000, 165: 3402–3410.**

Osteoarthritis (OA)[3] is a degenerative disease characterized by several structural changes including the degradation of cartilage matrix (1). In normal mature cartilage, chondrocytes synthesize sufficient amounts of macromolecules to maintain the integrity of the matrix, whereas in response to OA changes, they do not synthesize sufficient matrix to repair significant tissue defects (2). The chondrocyte is the only cell type found in mature cartilage and is responsible for the synthesis and the maintenance of the extracellular matrix. Therefore, factors that limit the adequate cartilage formation and repair may include the lack of chondrocytes in the tissue. There is a well-documented decline in the number of articular chondrocytes and an increase in the number of empty lacunae with age (3). Although some chondrocytes proliferate during OA, the chondrocytes do not migrate through the matrix to enter the site of tissue defect (2). Furthermore, there is increasing evidence suggesting that chondrocyte death may contribute to the progression of OA. Several studies have shown that OA cartilage has a higher number of apoptotic chondrocytes than does normal cartilage in animal models (4) and humans (5, 6). The presence of increased numbers of apoptotic cells may correlate with the extent of cartilage matrix loss (5).

The production of NO may represent an important component in the pathogenesis of OA. NO is produced in large amounts by chondrocytes upon proinflammatory cytokine stimulation (7). High levels of nitrite/nitrate have been found in the synovial fluid and serum of arthritis patients (8). Both mRNA and protein for inducible NO synthase (iNOS), the enzyme responsible for NO production, have also been detected in synovial tissue from OA patients (9). Besides causing degradation (10) or inhibiting the synthesis of cartilage matrix (11), NO may also induce chondrocyte apoptosis. We have previously reported that the systemic administration of iNOS inhibitor, *N*-iminoethyl-L-lysine (L-NIL), in experimentally induced OA in dogs has resulted in a reduction of articular cartilage damage and the levels of cell apoptosis and caspase-3, as determined immunohistochemically (12, 13). In addition, there is a significant correlation between the level of nitrite production and the prevalence of apoptotic cells in cartilage tissue during experimentally induced OA in rabbits (14). In fact, NO generated from sodium nitroprusside (SNP) has been shown to induce apoptosis in cultured human articular chondrocytes (15). However, the mechanisms regulating the chondrocyte death have not been well characterized.

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[3] Abbreviations used in this paper: OA, osteoarthritis; COX, cyclooxygenase; SNP, sodium nitroprusside; Z-DEVD-FMK, Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-CH_2F; Z-LEHD-FMK, Z-Leu-Glu(OCH_3)-His-Asp(OCH_3)-CH_2F; Z-DEVD-AFC, Z-Asp-Glu-Val-Asp-AFC; AFC, 7-amino-4-(trifluoromethyl)coumarine; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; ERK, extracellular signal-regulated kinase; iNOS, inducible NO synthase; L-NIL, *N*-iminoethyl-L-lysine; PDTC, pyrrolidine dithiocarbamate; L-NMMA, N^G-monomethyl-L-arginine; NSAIDs, nonsteroidal anti-inflammatory drugs.

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This study focused on the characterization of the signaling cascade during SNP-generated NO-induced cell death in human OA chondrocytes. We evaluated DNA fragmentation and cell viability to quantify the SNP-induced cell death in human OA chondrocyte culture and used various pharmacological inhibitors to study the different intracellular signaling pathways involved in this phenomenon. Caspase-3 activity and Bcl-2 level in the chondrocytes were also determined.

Materials and Methods

Materials

SNP, PGE_2, pyrrolidine dithiocarbanate (PDTC), and indomethacin were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada). Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-CH_2F (Z-DEVD-FMK), Z-Leu-Glu(OCH_3)-His-Asp(OCH_3)-CH_2F (Z-LEHD-FMK), PD98059, and SB202190 were the products of Calbiochem-Novabiochem (San Diego, CA). SN-50 and NS-398 were obtained from Biomol (Plymouth Meeting, PA) and Cayman Chemical (Ann Arbor, MI), respectively. All other chemicals were of the analytical grade of purity and commercially available.

Specimen selection and chondrocyte cultures

Cartilage specimens were obtained from 12 patients with OA (eight females, four males, aged 67 ± 9 years, mean ± SD) undergoing total knee joint replacement. Diagnosis was established according to the American College of Rheumatology criteria (16). The OA cartilage (femoral condyles and tibial plateaus), obtained under aseptic conditions, was carefully dissected from the underlying bone in each specimen. Approximately 2–5 g of cartilage were obtained from each dissected specimen. Gross morphology of the cartilage specimens used in this study was classified as moderate to severe OA.

Specimens were then dissected and washed in PBS containing antibiotics (500 U/ml penicillin, 500 μg/ml streptomycin) and again extensively washed in PBS. Chondrocytes were released from articular cartilage by sequential enzymatic digestion as described (17): 1 h with 2 mg/ml pronase (Boehringer Mannheim Canada, Laval, Quebec, Canada) followed by 18 h with 1 mg/ml collagenase (type IV; Sigma-Aldrich Canada) at 37°C in DMEM (Life Technologies, Canadian Life Technologies, Burlington, Ontario, Canada) with 10% heat-inactivated FCS (Life Technologies) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). The digested tissue was centrifuged and the pellet was washed. The isolated chondrocytes were seeded at high density in tissue culture flasks (no. 1-56502; Nunc, Roskilde, Denmark) and cultured in DMEM supplemented with 10% FCS (10% FCS-DMEM) and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At confluence, the cells were detached and passaged once, then seeded at 1×10^4 and 3×10^5 cells in a 96-well plate (Falcon 3072; Becton Dickinson, Franklin Lakes, NJ) and a 12-well plate (Costar 3513; Corning, Corning, NY), respectively. The cells were allowed to grow until confluence and then used in the following experiments.

Experimental culture conditions

SNP was used as a generator of NO. For the experiments on the SNP dose response, chondrocytes were treated with various concentrations of SNP for 24 h in 10% FCS-DMEM. To determine the time course of the response, cells were incubated with SNP (1 and 2 mM) for the indicated period (5–72 h).

To explore the signaling cascade on SNP-induced cell death, we used Z-DEVD-FMK (100 μM), Z-LEHD-FMK (100 μM), PD98059 (50 μM), SB202190 (10 μM), SN-50 (50 μg/ml), PDTC (10 μM), NS-398 (50 μM), and indomethacin (100 μg/ml). Chondrocytes were preincubated with each inhibitor for 2 h, followed by the coincubation of SNP (1 and 2 mM) for 24 h. Preliminary results confirmed that the effects of these inhibitors were dose dependent, and each inhibitor at the indicated concentration induced maximal response in our study (data not shown).

To examine the role of PGE_2 on SNP-mediated chondrocyte death, cells were first pretreated with various concentrations (1–1000 ng/ml) of PGE_2 for 48 h and then incubated with SNP (1 and 2 mM) in the absence of PGE_2 for an additional 24 h.

DNA fragmentation ELISA

To assay DNA fragmentation ELISA, chondrocytes were seeded at 1.0×10^4 per well in a 96-well culture plate in 100 μl 10% FCS-DMEM and cultured until confluence. Cells were then synchronized by 0.5% FCS-DMEM for 1 day. To label DNA, the medium was replaced with 10% FCS-DMEM and 10 μM 5-bromo-2′-deoxyuridine was added to each well and incubated for 20 h. Following 5-bromo-2′-deoxyuridine incorporation, the cells were cultured in 10% FCS-DMEM according to the experimental culture conditions as mentioned above. After the incubation, the cells were lysed in 200 μl incubation buffer (Roche Diagnostics, Laval, Quebec, Canada). Labeled DNA fragments were separated from labeled intact genomic DNA by centrifugation (10 min at 1000 × *g*). Soluble DNA fragments present in the supernatant were quantified using the Cellular DNA Fragmentation ELISA (Roche Diagnostics) according to the manufacturer's instructions. Results were expressed as OD units per 10^4 adherent cells.

Cell viability

Cell viability in a 96-well culture plate (see above) was evaluated using a modification of the MTT assay (18). For the colorimetric MTT assay, 10 μl MTT, a soluble tetrazolium salt solution (5 mg/ml in PBS), was added to the wells, containing 100 μl medium, and the plate was incubated for an additional 4 h. Thereafter, 100 μl solubilization solution (0.04 M HCl-isopropanol) was added to dissolve the water-insoluble formazan salt. Quantitation was then conducted with an ELISA reader at 590 nm. Results were expressed as OD units per 10^4 adherent cells.

Measurement of caspase-3 activity and Bcl-2 protein level

Chondrocytes were seeded at 3×10^5 cells per well in a 12-well culture plate in 2 ml 10% FCS-DMEM. After confluence, cells were treated with various concentrations of SNP for the indicated times.

For measurement of caspase-3 activity, adherent cells were washed with ice-cold PBS and resuspended in 100 μl lysis buffer (caspase-3 fluorometric assay; R&D Systems, Minneapolis, MN). The cell suspension was lysed by two cycles of freezing and thawing. Cell lysates (10 μg of total protein) were added to reaction mixtures containing 25 μM synthetic substrate Z-Asp-Glu-Val-Asp-AFC (Z-DEVD-AFC; Calbiochem-Novabiochem), 100 mM HEPES, 10% sucrose, 10 mM DTT, 1 mM PMSF, 10 μg/ml pepstatin, and 10 μg/ml leupeptin, pH 7.5, in a total volume of 100 μl. Caspase-3 activity was measured by the release of 7-amino-4-trifluoromethyl-coumarin (AFC) from the synthetic substrate Z-DEVD-AFC using a microplate spectrofluorometer in the kinetic mode with excitation and emission wavelengths of 400 and 505 nm, respectively. The reactions were inhibited by the addition of 100 μM Z-DEVD-FMK. The enzymatic activity was expressed in units per milligram of total protein, with 1 U corresponding to the amount of enzyme required to release 1 nmol AFC per min at 37°C. Protein content was determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL).

To assay the Bcl-2 protein level, adherent cells were washed with ice-cold PBS and resuspended in 100 μl 50 mM Tris, containing 5 mM EDTA, 0.2 mM PMSF, 1 μg/ml pepstatin, and 0.5 μg/ml leupeptin, pH 7.4. Ag Extraction Agent (20 μl; Oncogene Research Products, Cambridge, MA) was added, and the cell suspension was incubated on ice for 30 min to lyse the cells. Bcl-2 level in the cell lysate was assayed using Bcl-2 ELISA (Oncogene Research Products) according to the manufacturer's directions. The level of Bcl-2 was expressed in units per milligram of total protein, in which 1 U corresponded to the Bcl-2 protein level in 5.6×10^4 cells of HL60.

Western immunoblots for cyclooxygenase (COX)-2

Chondrocytes were seeded at 3×10^5 cells per well in a 12-well culture plate in 2 ml 10% FCS-DMEM and cultured until confluence. Cells were treated with various concentrations of SNP for 24 h. After this, the adherent cells were washed in ice-cold PBS once and cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1% Nonidet P-40, 1 mM sodium orthovanadate, and 1 mM NaF). The cell lysate was boiled for 5 min in 20 μl lysis buffer (1% SDS, 10 mM Tris, pH 7.4) and centrifuged for 5 min. The supernatant (10 μg protein) was subjected to SDS-PAGE through 9% gels (final concentration of acrylamide) under reducing conditions and transferred onto nitrocellulose membranes (Amersham, Oakville, Canada). After blocking with Superblock blocking buffer in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) and washing, the membranes were incubated overnight at 4°C with primary Ab in the blocking buffer as above and 0.5% Tween 20. The Ab used was a rabbit polyclonal anti-human COX-2 (1:5000 dilution; Cayman Chemical). A second anti-rabbit Ab (HRP conjugated, 1:20,000 dilution; Pierce) was subsequently incubated with membranes for 1 h at room temperature and then washed extensively (six times for 10 min each) with TTBS (20 mM Tris-HCl, 150 mM NaCl), pH 7.5, 0.1% Tween 20 at room temperature. Following incubation with the SuperSignal Ultra Chemiluminescent substrate (Pierce), membranes were prepared for autoradiography and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY).

Detection of nuclear lamin degradation

Expression of nuclear lamin and its degradation fragments were measured by Western blotting. Cells were lysed in 0.5% SDS, protein was determined, and Western immunoblots were performed as described above. After blocking, the membranes were incubated overnight at 4°C with mouse mAbs to lamin A and lamin C (a gift from Dr. Yves Raymond, Research Center, Center Hospitalier de l'Université de Montréal–Hôpital Notre-Dame, Montréal, Québec, Canada). A second anti-mouse Ab (HRP conjugated, 1:20,000 dilution; Pierce) was subsequently incubated with membranes for 1 h at room temperature and, finally, incubated with the SuperSignal Ultra Chemiluminescent substrate (Pierce).

PGE_2 production

PGE_2 was determined on the culture medium with the PGE_2 Enzyme Immunoassay Kit (Cayman Chemical). This assay uses the competition between PGE_2 and a PGE_2-acetylcholinesterase conjugate (PGE_2 tracer) for a limited amount of PGE_2 mAb. The sensitivity was 9 pg/ml, and the working range was between 10 and 1000 pg/ml, based on a logarithmic transformation.

Statistical analysis

All statistical analyses were accomplished using InStat Statistical Software (GraphPad, Sorrento Valley, CA). Results are expressed as mean ± SEM when at least three independent experiments were performed. Statistical comparisons were performed with an ANOVA followed by Dunnett's multiple comparison method. Values of $p < 0.05$ were considered statistically significant.

Results

SNP causes chondrocyte death, caspase-3 activation, and Bcl-2 down-regulation

Human OA chondrocytes were treated with the NO generator, SNP. The cell viability and the extent of nuclear DNA fragmentation were determined by the MTT assay and ELISA, respectively. Treatment with SNP for 24 h caused chondrocyte death in a dose-dependent manner (Fig. 1, *A* and *B*). Western blot analysis using antilamin A and C also confirmed that the cells contained the degradation fragments of nuclear lamin (data not shown), which is one of the characteristic changes during apoptosis (19). An initial 5-h exposure to SNP (1 and 2 mM) showed a significant increase in the extent of nuclear DNA fragmentation without any reduction in cell viability (Fig. 2, *A* and *B*).

Because caspase-3 is an executioner of apoptosis by a variety of stimuli (19), we examined whether SNP-generated NO activates caspase-3 in human OA chondrocytes. We also evaluated the effect of SNP on the level of apoptosis suppressor, Bcl-2, that is an intracellular protein and has been shown to enhance cell survival in part by inhibiting cytochrome *c* efflux from mitochondria, while protecting cells from apoptosis (20). The activity of caspase-3 and the level of Bcl-2 were assessed after treatment with SNP for 24 h. The treatment with SNP induced a dose-dependent increase in caspase-3 activity at the same time as a dose-dependent decrease in Bcl-2 level (Table I).

Effects of caspase, mitogen-activated protein kinase (MAPK), NF-κB, and COX inhibitors on SNP-induced chondrocyte death

To examine the signaling cascade on NO-induced cell death in human OA chondrocytes, we used various pharmacological inhibitors that affect different intracellular signaling. In this set of experiments, cells were preincubated with each inhibitor for 2 h, followed by the coincubation of SNP for 24 h. Cell death was initiated by the addition of 1 or 2 mM SNP and was analyzed on the extent of nuclear DNA fragmentation and cell viability. Neither DNA fragmentation nor cell viability in unstimulated controls was affected by each inhibitor used at indicated concentrations (Figs. 3–6).

To define the role of caspases on SNP-induced chondrocyte death, we used caspase-3 inhibitor Z-DEVD-FMK (100 μM) and caspase-9 inhibitor Z-LEHD-FMK (100 μM). Incubation of chon-

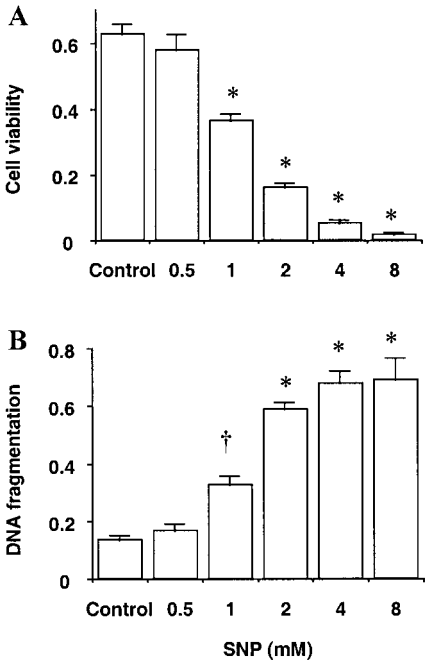

FIGURE 1. Dose-dependent effect of SNP on cell viability (*A*) and DNA fragmentation (*B*) in human OA chondrocytes. Cells were cultured in medium with or without various concentrations of SNP for 24 h. Mean ± SEM ($n = 6$). †, $p < 0.05$; ∗, $p < 0.01$ vs control; Dunnett's multiple comparison test.

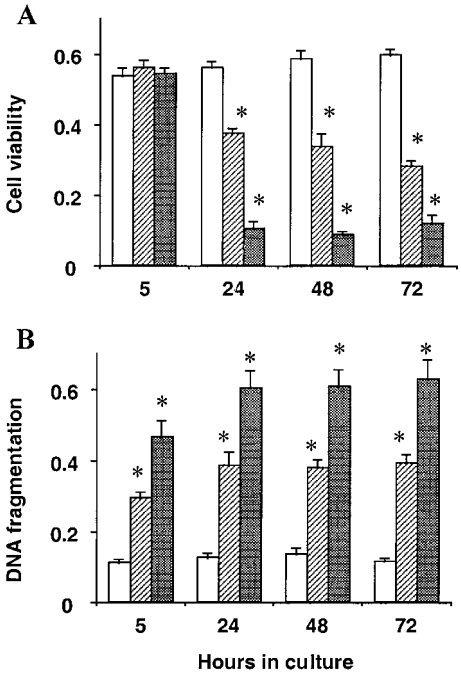

FIGURE 2. Time-dependent effect of SNP on cell viability (*A*) and DNA fragmentation (*B*) in human OA chondrocytes. Cells were cultured in medium with or without SNP (control, □; 1 mM, ▨; 2 mM, ■) for 5, 24, 48, and 72 h. Mean ± SEM ($n = 6$). ∗, $p < 0.01$ vs control at each indicated time; Dunnett's multiple comparison test.

Table I. *Dose-dependent effect of SNP on caspase-3 activity and Bcl-2 level in human OA chondrocytes*[a]

	Caspase-3 (U/mg protein)	Bcl-2 (U/mg protein)
Control	58.0 ± 10.6	0.13 ± 0.01
SNP (0.5 mM)	75.6 ± 11.8	0.12 ± 0.01
SNP (1 mM)	122.4 ± 15.3*	0.09 ± 0.01**
SNP (2 mM)	144.7 ± 20.7**	0.07 ± 0.01**

[a] Cells were cultured with or without various concentrations of SNP for 24 h. Caspase-3 activity and Bcl-2 level in adherent cells were assayed by Z-DEVD-AFC cleavage and ELISA, respectively, as outlined in *Materials and Methods*. Mean ± SEM ($n = 6$). *, $p < 0.05$; **, $p < 0.01$ vs control; Dunnett's multiple comparison test.

drocytes with the caspase inhibitors alone or in combination for 2 h followed by the subsequent addition of SNP (1 mM) totally prevented both SNP-mediated DNA fragmentation and reduction in cell viability (Fig. 3, *A* and *B*). Both DNA fragmentation and reduction in cell viability in response to 2 mM SNP were also prevented but partially by the addition of each or both caspase inhibitor (Fig. 3, *A* and *B*). These indicate that both SNP-initiated DNA fragmentation and reduction in cell viability depend on the activity of these caspases. In addition, combined treatment with Z-DEVD-FMK and Z-LEHD-FMK had no additive inhibitory effects on SNP-induced DNA fragmentation nor reduction in cell viability, suggesting that both caspase-3 and caspase-9 participate in the same sequence of cascade during NO-induced chondrocyte death.

To elucidate the role of the extracellular signal-regulated protein kinases (ERK)1/2 and p38 kinase during NO-mediated cell death, we interrupted ERK1/2 and p38 kinase signaling by using the MAPK kinase (MEK)1/2 inhibitor PD98059 (50 μM) and the p38 kinase inhibitor SB202190 (10 μM), respectively. As shown in Fig. 4, *A* and *B*, both PD98059 and SB202190 significantly inhibited DNA fragmentation in response to treatment with SNP, accompanied by an increase in cell survival. These results point to possible requirements of both ERK1/2 and p38 kinase during NO-elicited cell death.

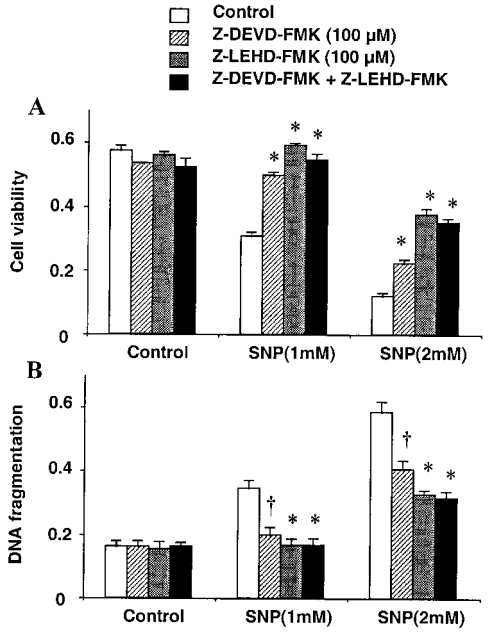

FIGURE 3. Effects of caspase inhibitors, Z-DEVD-FMK and Z-LEHD-FMK, on SNP-induced chondrocyte death. Cell viability (*A*) and DNA fragmentation (*B*). Cells were pretreated with Z-DEVD-FMK (100 μM), Z-LEHD-FMK (100 μM), or Z-DEVD-FMK (100 μM) + Z-LEHD-FMK (100 μM) for 2 h, followed by the coincubation of SNP (1 and 2 mM) for an additional 24 h. Mean ± SEM ($n = 6$). †, $p < 0.05$; ∗, $p < 0.01$ vs control without each inhibitor; Dunnett's multiple comparison test.

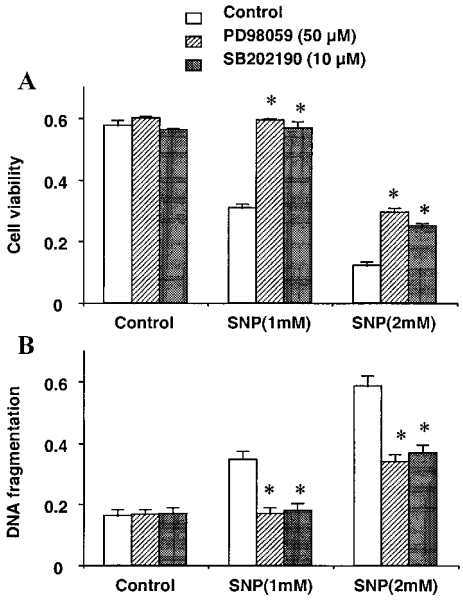

FIGURE 4. Effects of MAPK inhibitors, PD98059 and SB202190, on SNP-induced chondrocyte death. Cell viability (*A*) and DNA fragmentation (*B*). Cells were pretreated with PD98059 (50 μM) or SB202190 (10 μM) for 2 h, followed by the coincubation of SNP (1 and 2 mM) for an additional 24 h. Mean ± SEM ($n = 6$). ∗, $p < 0.01$ vs control without each inhibitor; Dunnett's multiple comparison test.

We also tested the effects of NF-κB inhibitors, SN-50 (cell-permeable inhibitory peptide) and PDTC, because this transcription factor has also been implicated in the regulation of apoptosis (21). Treatment with SN-50 prevents nuclear translocation of the activated NF-κB complex (22), whereas PDTC inhibits NF-κB activation (23). SN-50 (50 μg/ml) tended to enhance DNA fragmentation and reduce cell viability following SNP addition, but this effect was not significant (Fig. 5, *A* and *B*). A control peptide for SN-50 (SN-50 M, 50 μg/ml) had no effect (data not shown). PDTC (10 μM) significantly enhanced DNA fragmentation with a further reduction in cell viability (Fig. 5, *A* and *B*). This implies an apoptosis-enhancing capability of the NF-κB inhibitor PDTC.

Because NO has been shown to stimulate PG biosynthesis in vitro and in vivo (24, 25), we also examined the effects of the COX-2-specific inhibitor NS-398 and the COX-1/COX-2 inhibitor indomethacin on SNP-induced chondrocyte death. Incubation of chondrocytes with NS-398 (50 μM) for 2 h followed by the subsequent coincubation of SNP (1 mM) completely blocked both SNP-induced DNA fragmentation and reduction in cell viability (Fig. 6, *A* and *B*). Both DNA fragmentation and reduction in cell viability in response to 2 mM SNP were also inhibited by NS-398, but this inhibitory effect was less marked (Fig. 6, *A* and *B*). NS-398 at this concentration caused maximal response regarding inhibition of the cell death (data not shown). Treatment with 100 μg/ml indomethacin was equally effective in attenuating both SNP-mediated DNA fragmentation and reduction in cell viability, as was NS-398 treatment (Fig. 6, *A* and *B*). These data show that COX-2 appears to be one of the key regulators of NO-induced cell death in human OA chondrocytes.

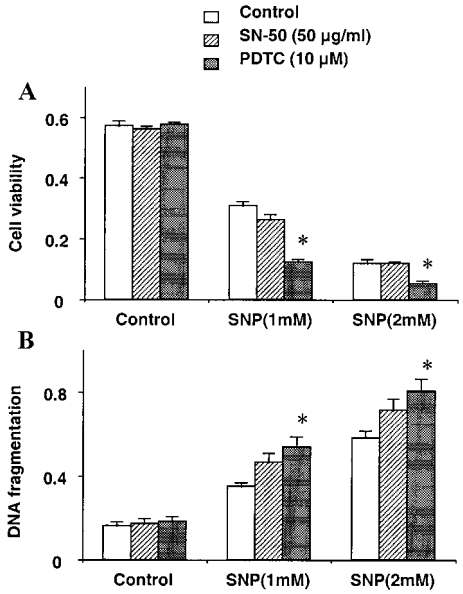

FIGURE 5. Effects of NF-κB inhibitors, SN-50 and PDTC, on SNP-induced chondrocyte death. Cell viability (*A*) and DNA fragmentation (*B*). Cells were pretreated with SN-50 (50 μg/ml) or PDTC (10 μM) for 2 h, followed by the coincubation of SNP (1 and 2 mM) for an additional 24 h. Mean ± SEM ($n = 6$). ∗, $p < 0.01$ vs control without each inhibitor; Dunnett's multiple comparison test.

SNP induces COX-2 expression and PGE_2 production in chondrocytes

To clarify whether SNP-generated NO induces COX-2 expression in human OA chondrocytes, we examined the level of COX-2 protein by Western blot analysis. SNP induced COX-2 expression and PGE_2 release in a dose-dependent manner (Fig. 7). The COX-2 protein was not expressed in unstimulated controls.

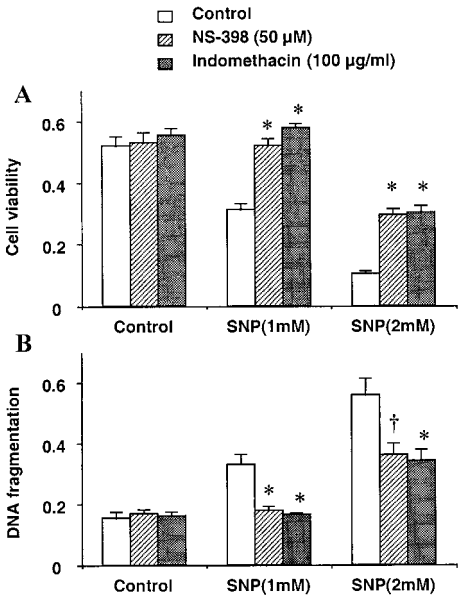

FIGURE 6. Effects of COX inhibitors, NS-398 and indomethacin, on SNP-induced chondrocyte death. Cell viability (*A*) and DNA fragmentation (*B*). Cells were pretreated with NS-398 (50 μM) or indomethacin (100 μg/ml) for 2 h, followed by the coincubation of SNP (1 and 2 mM) for an additional 24 h. Mean ± SEM ($n = 6$). †, $p < 0.05$; ∗, $p < 0.01$ vs control without each inhibitor; Dunnett's multiple comparison test.

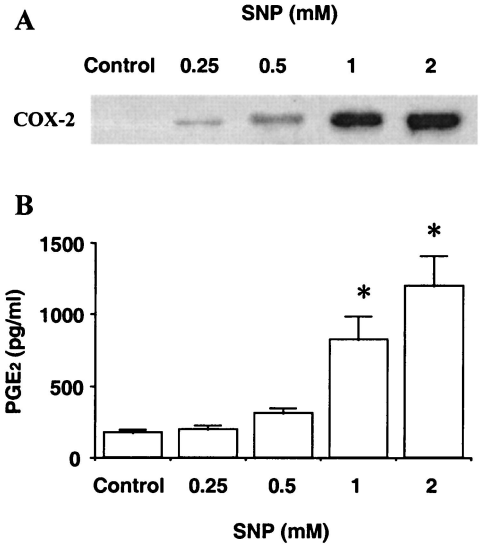

FIGURE 7. Dose-dependent effect of SNP on COX-2 expression (*A*) and PGE_2 production (*B*). Cells were cultured with or without various concentrations of SNP for 24 h. The COX-2 expression in adherent cells was determined by Western blotting. PGE_2 released into culture medium was measured by enzyme immunoassay. Mean ± SEM ($n = 6$). ∗, $p < 0.01$ vs control; Dunnett's multiple comparison test.

To evaluate the relationship between caspases, MAPK, and PGE_2 synthesis during SNP-induced cell death in chondrocytes, we measured PGE_2 release after treatment of chondrocytes with SNP (1 mM) in the presence or absence of Z-DEVD-FMK (100 μM), Z-LEHD-FMK (100 μM), PD98059 (50 μM), SB202190 (10 μM), NS-398 (50 μM), or indomethacin (100 μg/ml). The MEK1 inhibitor PD98059, the p38 kinase inhibitor SB202190, the COX-2 specific inhibitor NS-398, and the COX-1/COX-2 inhibitor indomethacin totally blocked the PGE_2 release response to 1 mM SNP (Table II). Neither the caspase-3 inhibitor Z-DEVD-FMK nor the caspase-9 inhibitor Z-LEHD-FMK had any effect on the PGE_2 production (Table II). Both PD98059 and SB202190 also inhibited the SNP-induced COX-2 expression (data not shown). This suggests that the ERK1/2 and p38 kinase pathways are upstream signaling of the PGE_2 production, whereas the caspase cascade is not involved in PGE_2 production during NO-induced cell death in human OA chondrocytes.

PGE_2 sensitizes chondrocytes to the cell death-inducing effect of NO

To determine the role of PGE_2 on SNP-induced chondrocyte death, cells were pretreated with various concentrations of PGE_2 (1–1000

Table II. *Effect of caspase inhibitors, MAPK inhibitors, and COX inhibitors on SNP-mediated PGE_2 production in human OA chondrocytes*[a]

	PGE_2 (pg/ml)
Control	176.3 ± 25.2
SNP (1 mM)	1177.7 ± 320.4*
SNP (1 mM) + Z-DEVD-FMK (100 μM)	957.3 ± 248.2*
SNP (1 mM) + Z-LEHD-FMK (100 μM)	830.8 ± 271.0*
SNP (1 mM) + PD98059 (50 μM)	62.0 ± 9.0†
SNP (1 mM) + SB202190 (10 μM)	48.0 ± 6.4†
SNP (1 mM) + NS-398 (50 μM)	62.3 ± 7.8†
SNP (1 mM) + indomethacin (100 μg/ml)	77.0 ± 8.1†

[a] Cells were pretreated with each inhibitor for 2 h, followed by the coincubation of SNP (1 mM) for an additional 24 h. PGE_2 released into culture medium was measured by enzyme immunoassay. Mean ± SEM ($n = 4$). *, $p < 0.01$ vs control; †, $p < 0.01$ vs SNP (1 mM); Dunnett's multiple comparison test.

ng/ml) for 48 h followed by a subsequent incubation of SNP (1 and 2 mM) without PGE_2. As shown in Fig. 8, *A* and *B*, pretreatment with PGE_2 significantly enhanced the sensitivity of chondrocytes to both SNP-induced DNA fragmentation and reduction in cell viability. PGE_2 alone did not induce chondrocyte death. Pretreatment of PGE_2 for 24 h was long enough to cause its effect (data not shown). In addition, treatment with PGE_2 affected neither the caspase-3 activity (control, 65.5 ± 15.6 U/mg protein; PGE_2, 71.2 ± 20.1 U/mg protein, mean ± SEM, $n = 6$) nor the Bcl-2 level (control, 0.126 ± 0.014 U/mg protein; PGE_2, 0.122 ± 0.013 U/mg protein, mean ± SEM, $n = 6$). These data suggest that exogenous PGE_2 sensitizes human OA chondrocytes to the cell death-inducing effect of NO, and the mechanisms underlying the effect of PGE_2 does not link directly to caspase-3 activity and Bcl-2 level.

Discussion

In this study, we demonstrated that SNP-generated NO caused chondrocyte death through COX-2-mediated PGE_2 production. Blanco et al. (15) have reported that SNP-generated NO-induced apoptosis in cultured human chondrocytes as determined by electron microscopy, 4′,6-dianidino-2-phenylindole dihydrochloride staining, flow cytometry, and histochemical detection of DNA fragmentation. We also supported these data pharmacologically, that is, both the caspase-3 inhibitor Z-DEVD-FMK and the caspase-9 inhibitor Z-LEHD-FMK completely blocked the SNP (1 mM)-induced chondrocyte death, suggesting that the cell death depends on the activity of caspases, which are largely absent in necrotic cells (26). In fact, the SNP-mediated chondrocyte death was accompanied by an increase in the activity of caspase-3. In contrast, these caspase inhibitors did not affect the chondrocyte death induced by hydrogen peroxide (Notoya et al., unpublished observation), which has been shown to induce necrosis in human chondrocytes (15). Therefore, this report provides a possible explanation for mechanisms by which NO induces apoptosis in human OA chondrocytes. However, a high dose of SNP (2 mM) also caused caspase-independent cell death, probably due to primary or secondary necrosis (27), although part of the chondrocyte death still depended on the activity of caspases.

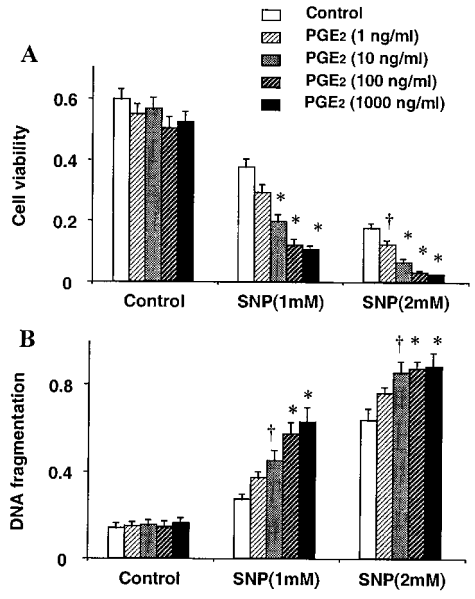

FIGURE 8. Sensitization of SNP-mediated chondrocyte death by PGE_2 pretreatment. Cells were pretreated with or without various concentrations of PGE_2 for 48 h, followed by a subsequent incubation of SNP (1 and 2 mM) without PGE_2. Mean ± SEM ($n = 6$). †, $p < 0.05$; ∗, $p < 0.01$ vs control without PGE_2 pretreatment; Dunnett's multiple comparison test.

SNP-generated NO is capable of stimulating the production of PGE_2 via the induction of COX-2 in human OA chondrocytes. Our results parallel the studies by Hughes et al. (28) and by Kenthen and Brune (29) that indicate NO donors induced the expression of COX-2 protein as observed in osteoblasts and macrophages, respectively. In contrast, other studies show that NO may inhibit PGE_2 release. Stadler et al. (30) have demonstrated that treatment with LPS and IFN-γ up-regulates the production of both NO and PGE_2 in rat Kupffer cells. In these cells, the inhibition of NO production by a nonselective NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA), further increased PGE_2 production. This finding is consistent with the studies of Henrotin et al. (31) and Amin et al. (32), who have demonstrated that L-NMMA enhanced PGE_2 production in cytokine-stimulated human chondrocytes and cartilage explants, respectively. However, experiments conducted in our laboratory have shown that L-NIL, a selective inhibitor of iNOS, had no effect on the level of PGE_2 production by IL-1-stimulated human OA chondrocytes (Pelletier et al., unpublished observation). These variances may be related to the balance of various mediators other than NO, which also affect the expression of COX-2 and/or the production of PGE_2 in the microenvironment of these cultures. For instance, in the study of Henrotin et al. (31), the inhibition of NO synthase by L-NMMA led to the enhancement not only of PGE_2 but also of other cytokines, such as IL-6 and IL-8, in human chondrocytes stimulated by IL-1β or in combination with LPS. Both IL-6 and IL-8 are also capable of up-regulating the expression of COX-2 (33, 34), whereas our data, like data from the study of Blanco and Lotz using human normal chondrocytes (35), indicated the intrinsic positive effect of NO on PGE_2 production in human OA chondrocytes without the influence of high levels of cytokines.

With respect to mechanisms underlying the PGE_2 production enhanced by NO, we demonstrated that both the MEK1/2 inhibitor PD98059 and the p38 kinase inhibitor SB202190 abolished the SNP-induced PGE_2 production as well as COX-2 expression. This suggests that both ERK1/2 and p38 kinase are possible mediators during this process. Shalom-Barak et al. (36) have reported that the p38 kinase inhibitor SB203580 prevented COX-2 expression induced by IL-17 in human normal chondrocytes, indicating the p38 kinase pathway may be linked to the induction of COX-2 in these cells. In addition, overexpressing ERK1, c-Jun N-terminal kinase, or p38 kinase leads to severalfold increases in COX-2 promoter activity in human mammary epithelial cells (37). Biochemical analysis of MAPK during NO-induced chondrocyte death is now under investigation. Besides the induction of COX-2, NO, either directly or indirectly through peroxynitrite, may interact with COX to cause an increase in enzymatic activity (24, 38).

COX-2-mediated PGE_2 production is required for caspase-dependent chondrocyte death induced by NO, because both the inhibition of COX-2 by NS-398 and the inhibition of COX-1/COX-2 by indomethacin totally attenuated SNP (1 mM)-mediated cell death in human OA chondrocytes. Similarly, Pasinetti and Aisen (39) have demonstrated that up-regulation of COX-2 expression overlapped the cellular morphological features of apoptosis in frontal cortex of Alzheimer's disease brain. Indeed, in a neuroectodermal cell line P19 cells, the induction of COX-2 precedes apoptosis in response to serum deprivation (40). Moreover, the inhibition of PGE_2 synthesis by indomethacin leads to cell survival in vascular smooth muscle cells of abdominal aortic aneurysms

(41). In contrast, several studies in the literature indicate that COX-2 expression plays a role in preventing apoptosis in a number of cancer cells and macrophages (42). For example, rat intestinal epithelial cells overexpressing COX-2 have enhanced ability to bind to extracellular matrix proteins and are resistant to undergoing apoptosis (43). Also, COX-2-overexpressing macrophages reveal protection of NO-mediated apoptosis (29). Furthermore, the inhibition of COX-1/COX-2 by nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to induce apoptosis in human colorectal cancer cells (44) and to be capable of reducing colon tumor (45). Thus, the relationship between COX-2 expression and cell death seems to be tissue specific. It may depend on a variety of PGE_2-induced cellular responses through PGE receptor subtypes and isoforms, which express and function in specific tissues and cells (46). In addition, the intracellular turnover of sphingomyelin may offer a possible explanation for a tissue-specific role of COX for cell death. Regarding mechanisms underlying NSAID-mediated apoptosis in colon tumor cells, the study of Chan et al. (44) suggests that the inhibition of COX-1/COX-2 by NSAIDs results in an increase in the cellular pool of arachidonic acid, and this, in turn, stimulates the conversion of sphingomyelin to ceramide, which promotes apoptosis. Although exogenous cell-permeable ceramide causes chondrocyte apoptosis (47), our preliminary data indicated that fumonisin B1, an inhibitor of ceramide generation, did not affect SNP-induced chondrocyte death, indicating that ceramide generation seems to be independent from NO-induced chondrocyte death.

PGE_2 enhanced the cell death induced by NO in human OA chondrocytes. Because caspase inhibitors, Z-DEVD-FMK and Z-LEHD-FMK, could abolish the SNP (1 mM)-mediated cell death without affecting PGE_2 production, endogenous PGE_2 is necessary but not sufficient for caspase-dependent chondrocyte death induced by NO. Our results also demonstrated that PGE_2 itself did not induce chondrocyte death. Likewise, we showed that PGE_2 itself influenced neither caspase-3 activity nor the level of Bcl-2 in human OA chondrocytes. Moreover, several studies indicate that PGE_2 does not alter NOS expression and the enzyme activity in chondrocytes (35). Consequently, PGE_2 may decrease apoptotic thresholds of the chondrocytes against NO-induced cytotoxicity, including direct DNA damage, the generation of peroxynitrite, and the inactivation of antioxidant enzymes (48–50). The mechanisms by which PGE_2 sensitized human OA chondrocyte to cell death induced by NO are not clear, but might be related to varying the set point for apoptosis through apoptosis inducers such as c-Myc and the adenovirus oncoprotein E1A, which lower the threshold for apoptosis induction under a variety of conditions (51). Indeed, histological analysis using the rabbit growth plate indicates that there is an increased colocalization of c-Myc with TUNEL-positive chondrocytes with age (52). Furthermore, Pica et al. have reported that PGE_2 stimulated the expression of c-Myc protein, but did not affect Bcl-2 protein levels in human lymphocytes (53). In addition to this, regulation of inactive caspase levels may also be related to the PGE_2-mediated sensitization of chondrocytes to the cell death induced by NO. For example, TNF-α regulates the Fas-mediated apoptosis signaling via an up-regulation of inactive caspase levels, such as caspase-3 and caspase-8, in synovial cells (54).

A recent study using bovine normal articular chondrocyte has demonstrated that even PGE_2 itself induced DNA fragmentation of the cells, as determined by ELISA (55). This controversy may be due to differences in culture conditions and/or cartilage specimen used for cell culture. Results of further experimental studies are needed to determine whether there are some altered apoptotic responses in chondrocytes from OA vs normal cartilage. A similar issue could be raised regarding normal and OA synoviocytes. Work in progress in our laboratory indicates that OA synoviocyte apoptosis can be induced under experimental conditions similar to those used in the actual study with OA chondrocytes. Therefore, these findings indicate that the mechanism reported in our study does not seem to be unique to OA chondrocytes.

The schematic summary of this data is shown in Fig. 9. SNP-generated NO induces COX-2 expression, possibly through ERK1/2 and p38 kinase pathways, resulting in an increase in release of PGE_2 in human OA chondrocytes. And by autocrine/paracrine mechanisms, PGE_2 may sensitize chondrocytes to the cell death induced by NO. Thus, both NO and PGE_2, two pleiotropic mediators in arthritis, play a crucial role in chondrocyte death. This phenomenon in vitro might occur and be significant in the progression of OA. In fact, our recent reports using the experimental OA dogs have demonstrated that an inhibition of high levels of NO production by L-NIL caused a decrease in COX-2 protein expression in articular cartilage, resulting in a reduction of the total amount of PGE_2 in synovial fluid (56). L-NIL itself has no direct inhibitory action on COX-2 activity and PG isomerase activity (25). Therefore, this finding suggests the positive influence of NO on PGE_2 synthesis in synovial joints of OA, particularly in the early stage of the disease, because the lesions observed in the experimental model are mild to moderate in severity. Also, the systemic administration of L-NIL reduced both the level of chondrocyte apoptosis and the progression of OA (12, 13). Additional studies will determine whether COX inhibitors can prevent chondrocyte apoptosis in OA cartilage in vivo. It seems likely that the intracellular signaling during NO-induced chondrocyte death, such as MAPK and caspase cascade, is also the possible molecular target for inhibition of chondrocyte apoptosis, and further research on the regulation of chondrocyte apoptosis, including implication of the cytokine network, will provide new concepts for treatment of

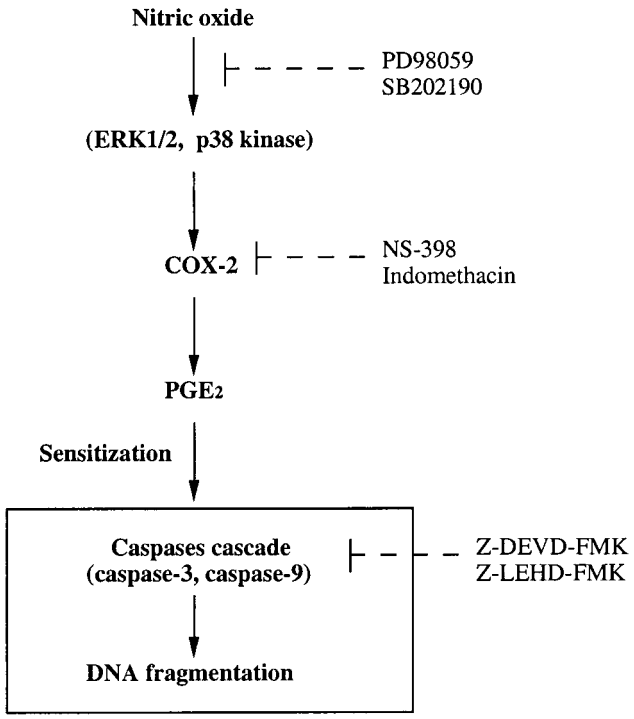

FIGURE 9. Schematic summary of the NO-induced cell death in human OA chondrocytes. Broken lines show the paths of action of the MAPK inhibitors (PD98059 and SB202190), COX inhibitors (NS-398 and indomethacin), and caspase inhibitors (Z-DEVD-FMK and Z-LEHD-FMK).

OA. In addition, selective cellular regulation of apoptosis in synovial joints might be required for this therapeutic strategy in arthritis (57).

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