Nitric Oxide Induced Cell Death in Human Osteoarthritic Synoviocytes Is Mediated by Tyrosine Kinase Activation and Hydrogen Peroxide and/or Superoxide Formation

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ABSTRACT. ***Objective.*** To investigate the regulation of osteoarthritis (OA) synovial fibroblast nitric oxide (NO) induced cell death.

Methods. Cultured synovial fibroblasts from human OA synovium were incubated with NO donor sodium nitroprusside (SNP) in the absence or presence of specific inhibitors of different protein kinases, cyclooxygenase-2 (COX-2), caspase-3 and caspase-9, inducible NO synthase, and in the absence or presence of prostaglandin E_2 (PGE_2). Experiments were also performed using scavengers of NO (carboxy-PXTO), peroxynitrite (uric acid), and superoxide (taxifolin). The level of cell death was measured by MTT and DNA fragmentation.

Results. Human OA synovial fibroblasts incubated with SNP decreased cell viability and increased DNA fragmentation in a dose dependent manner. This was associated with increased levels of both COX-2 and PGE_2 production. Selective inhibition of COX-2 by NS-398 significantly inhibited SNP induced cell death, even in the presence of exogenously added PGE_2. Experiments revealed that SNP treated cells expressed increased levels of active caspase-3 and caspase-9, while Bcl-2 was down-regulated. Incubation of these treated cells with inhibitors of caspase-3 (Z-DEVD-FMK) or caspase-9 (Z-LEHD-FMK) protected viability of SNP treated OA synovial fibroblasts, indicating that NO mediated cell death was mainly related to apoptosis. This was also confirmed by measuring the DNA fragmentation (TUNEL method) and the level of active caspase-3 (immunocytochemistry) in these cells. Data also showed that SNP induces the activation of kinases MEK 1/2, p38, and tyrosine kinases. Specific inhibition of tyrosine kinases completely abrogated the SNP induced cell death. In turn, this cell death protection was associated with a marked inhibition of caspase-3 and caspase-9 activities, as well as COX-2/PGE_2 production. Moreover, data showed that the NO donor SNP induced cell death was not solely related to the production of NO or peroxynitrite, but to the generation of reactive oxygen species (ROS) such as hydrogen peroxide and/or superoxide.

Conclusion. Our results provided strong evidence of the role of tyrosine kinase and mitogen activated protein kinase activation, by upregulation of COX-2 expression, in NO induced OA synovial fibroblast death. The generation of ROS such as hydrogen peroxide and superoxide appeared to be a major factor in the death of these cells. (J Rheumatol 2002;29:2165–75)

Key Indexing Terms:
OSTEOARTHRITIS SYNOVIOCYTES APOPTOSIS
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Osteoarthritis (OA) is the most common musculoskeletal degenerative disease characterized by morphological and biochemical alterations of articular cartilage and synovial membrane[1]. Even in the early stages, increases in enzymatic activity and morphological changes are found in the synovial membrane in OA[2]. Prominent synovial inflammation next to the area of cartilage lesions is often observed during the course of the disease[3], which probably plays an important role in the pathogenesis of OA[1]. OA synovial membranes produce a significant amount of metalloproteinases and proinflammatory cytokines such as tumor necrosis factor-α and interleukin 1ß (IL-1ß)[1] that could significantly affect cartilage and synovium metabolism.

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Implication of nitric oxide (NO) in joint disease[4,5] has been suggested because substantial concentrations of NO metabolites such as nitrite, nitrate, and nitrosoproteins are found in human OA and rheumatoid arthritis synovial fluids[6,7]. The excessive level of production of NO by OA tissues may represent an important component in the pathogenesis of OA[1,6,7]. The involvement of NO in the induction of chondrocyte death in human OA cartilage has been described by Blanco, *et al*[8]. There are several potential sources of NO in the OA joint; the chondrocytes have been characterized as a major intraarticular cell source[7,9]. Cultures of human chondrocytes synthesize large amounts of NO in response to stimulation by IL-1ß[10]. NO is a highly diffusible radical that can theoretically exert long-lasting intracellular action on synovial cells[7], even though its half-life is relatively short[11]. In experimentally induced OA in dogs, systemic administration of the inducible form of NO synthase (iNOS) inhibitor, *N*-imonoethyl-L-lysine (L-NIL), has been reported to reduce articular cartilage damage, as well as the levels of chondrocyte death and caspase-3[12,13]. These latter findings support the role of NO in the pathophysiology of OA.

We have reported that NO generated from sodium nitroprusside (SNP) induced cell death in human OA chondrocytes via the induction of prostaglandin E_2 (PGE_2) synthesis[14]. Our aims in this study were to investigate the potential role of NO in modulation of the viability of OA synoviocytes, to characterize the signaling cascade mediating the effects of NO, and to explore the mechanisms by which SNP could induce cell death.

MATERIALS AND METHODS

Materials. Sodium nitroprusside, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), sodium thiosulfate, and PGE_2 were purchased from Sigma-Aldrich (Oakville, ON, Canada). NS-398 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp (OCH_3)-CH_2F (Z-LEHD-FMK), Z-Leu-Glu (OMe)-His-Asp (Ome)-CH_2F (Z-LEHD-FMK), KT 5926, CalC, KT 5720, uric acid, genistein, cyclosporine A, taxifolin, carboxy-PTIO, BPIQ-II, AG-490, SB-202190, and PD-98059 were the products of Calbiochem-Novabiochem (San Diego, CA, USA). N-imonoethyl-L-lysine (L-NIL) was obtained from Pharmacia Corp. (St. Louis, MO, USA). All chemicals were analytical grade of purity.

Cell preparation. Synovial membranes were obtained from 34 patients with OA (21 women, 13 men, mean age 62.4 ± 3.8 yrs) undergoing total knee replacement. All patients were evaluated, and diagnosis was based on criteria of the American College of Rheumatology[15]. For synovial cell isolation, finely cut synovium was digested as described[16]. The isolated cells were seeded at high density in tissue culture flasks (Nalge Nunc International, Naperville, IL, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL Canadian Life Technologies, Burlington, ON, Canada) containing 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco-BRL Canadian Life Technologies), and supplemented with 10% heat inactivated fetal calf serum (FCS; Gibco-BRL Canadian Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At confluence, cells were detached and passaged once, then seeded at 50×10^3, 4×10^5, and 8×10^5 cells in a 96 well plate (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA), 12 well plate (Costar 3513; Corning, Corning, NY, USA) and 6 well plate (Nalge Nunc International), respectively. Second or third passage cells were allowed to grow to confluence and used in the following experiments.

Experimental culture conditions. Because of its ability to induce apoptosis in synoviocytes, SNP, a generator of NO, was chosen as a proapoptotic agent[17,18]. Synoviocytes (5×10^4 cells/well) were treated with SNP (0–2 mM) for 18 h in 10% FCS-DMEM. To explore the involvement of different protein kinases on signaling cascades in SNP induced cell death, we used CalC (250 nM), KT-5720 (1 µM), KT-5926 (1 µM), SB-202190 (10 µM), and PD-98059 (50 µM). Experiments were performed using genistein (0–50 µM), AG-490 (0–25 µM), and BPIQ-II (0–20 µM) as tyrosine kinase inhibitors. These different inhibitors were tested up to the maximal concentrations that were possible under the actual experimental conditions without inducing cytotoxicity. To examine the role of endogenously synthesized PGE_2 and NO, and the role of exogenously added PGE_2, we used NS-398, (0–50 µM), L-NIL (50 µM), and PGE_2 (0–1000 ng/ml), respectively. To show the role of caspase-3 and caspase-9 in NO induced synoviocyte death, we used specific inhibitors of the 2 enzymes, Z-DEVD-FMK (0–100 µM) and Z-LEHD-FMK (0–100 µM), respectively. The maximum concentrations tested were those found to be the highest that could possibly be used under these experimental conditions.

To explore the effect of oxidative stress/free radicals in NO mediated synoviocyte apoptosis, we used scavengers for peroxynitrite ($ONOO^-$) (uric acid, 0–2 mM), NO (carboxy-PTIO, 0-200 µM), and superoxide (O_2^-)-hydrogen peroxide (H_2O_2) (taxifolin, 0–100 µM) and cyanide (sodium thiosulfate, 0–25 mM). The release of mitochondrial cytochrome C into the cytoplasm was blocked using cyclosporine A (0–100 µM).

Recognition of DNA strand breaks by the terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) method and active caspase-3 by immunocytochemistry. DNA strand breaks and/or active caspase-3 were visualized in cells using a double labeling kit (R&D Systems, Minneapolis, MN, USA). Cells treated for 18 h with 0.5 mM SNP were fixed on slides with 1% paraformaldehyde for 10 min, then permeabilized by postfixing for 5 min in precooled ethanol:acetic acid 2:1. Cells were processed for TUNEL reaction and active caspase-3 immunochemistry (rabbit anti-active caspase-3 antibody, dilution 1/40, catalog #AF835) as specified by the manufacturer's instructions. Specimens were counterstained with 0.5% (w/v) methyl green and analyzed microscopically. To determine the specificity of staining, 3 control procedures were used, according to the same experimental protocol: (1) use of adsorbed immune serum (1 h at 37°C) with 20-fold molar excess of recombinant or purified antigen; (2) omission of the primary antibody; and (3) substitution of the primary antibody with an autologous preimmune serum. The purified antigen used in our study was human recombinant caspase-3 (Upstate Biotechnology, Lake Placid, NY, USA).

Cyclooxygenase (COX-2) Western immunoblot. For COX-2 detection, synoviocytes 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) and 10 µg supernatant proteins were loaded onto a 9% SDS-PAGE gel and transferred onto nitrocellulose membrane (Amersham, Baie d'Urfé, QC, Canada). The primary antibody for COX-2 detection was a rabbit polyclonal anti-human COX-2 (1:5000 dilution; Cayman Chemical), and the second anti-rabbit antibody was HRP conjugated (1:20,000 dilution; Pierce Chemical, Rockford, IL, USA). After incubation with SuperSignal Ultra chemiluminescence substrate (Pierce), membranes were prepared and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY, USA).

Determination of mitochondrial membrane potential ($\Delta\Psi_m$). The mitochondrial membrane potential of synoviocytes, in the absence or presence of NO donor (0.5 mM SNP, 16 h), was detected using a potential-dependent, aggregate-forming lipophilic cation, JC-1 (Molecular Probes Inc., Eugene, OR, USA). JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetra-ethylbenzimidazolycarbocyanine iodide) is a cationic dye that exhibits potential-dependent accumulation in mitochondria; a fluorescence emission shift

from red (595 nm) to green (525 nm) corresponds to aggregate and monomer forms of the dye. Synoviocytes were incubated 10 min with JC-1 (0.3 μg/ml). The cells were analyzed by fluorescence microscopy (Nikon Eclipse E 600) or flow cytometry (FACScan, Becton Dickinson, Palo Alto, CA, USA).

p53 and Bcl-2 detection. Measurements of p53 and Bcl-2 in cell lysate were performed using p53 and Bcl-2 ELISA kits (Roche Diagnostics, Laval, QC, Canada; Oncogene Research Products, San Diego, CA, USA, respectively) according to the manufacturer's instructions. The limit of detection of the assay was 9 pg/ml for p53 and 1 unit/ml for Bcl-2.

Statistics. Values were expressed as mean ± SEM, and *n* refers to the number of different cell lines (i.e., donors). Statistical significance was assessed using the Student t test. $P < 0.05$ was considered significant.

RESULTS

SNP causes synoviocyte death, caspase-3 activation and Bcl-2 downregulation. Human OA synoviocytes were treated with the NO generator SNP because of its ability to induce OA synoviocyte apoptosis[14,17,18]. Cell viability and the extent of nuclear DNA fragmentation were determined by the MTT[19] and ELISA assays, respectively. Treatment with SNP for 18 h caused synoviocyte death and DNA fragmentation in a dose dependent manner (Figure 1). These data were also corroborated by TUNEL reaction on cultured synovial cells and showed a marked increase in the number of synovial cells staining positive (Figures 2A, 2B). Addition of sodium thiosulfate (0–25 mM) in the culture medium had no effect on the induction of synovial cell death by SNP, indicating that cyanide released by SNP had no effect on the level of cell death (data not shown).

Since caspases carry out apoptosis by a variety of stimuli, we examined whether SNP generated NO activated caspase-3 in human OA synoviocytes. We also evaluated the effect of SNP on the level of the apoptosis suppressor Bcl-2, an intracellular protein that 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 18 h. The treatment with SNP induced an increase in caspase-3 and caspase-9 activity at the same time as a decrease in the concentration of Bcl-2 (Table 1). Similar results were obtained by immunocytochemistry, whereas apoptotic synoviocytes were also shown to be positive for active caspase-3 (Figures 2C, 2D). Controls showed only background staining (data not shown).

Endogenously produced PGE_2 necessary for NO induced synoviocyte death. The effect of endogenously produced and exogenously added PGE_2 on SNP induced (0.5 mM, 18 h) synoviocyte death was examined (Figure 3A). The COX-2 specific inhibitor NS-398 (0–50 μM) dose dependently and significantly ($p < 0.02$) protected synoviocytes against the cytotoxic effect of NO, which implied that the endogenous production of PGE_2 is essential for NO induced synoviocyte death. The addition of exogenous PGE_2 (0–1000 ng/ml) alone or in the presence of NS-398 had no effect on viability. Selective inhibition of iNOS by L-NIL had no effect on the NO induced synoviocyte death.

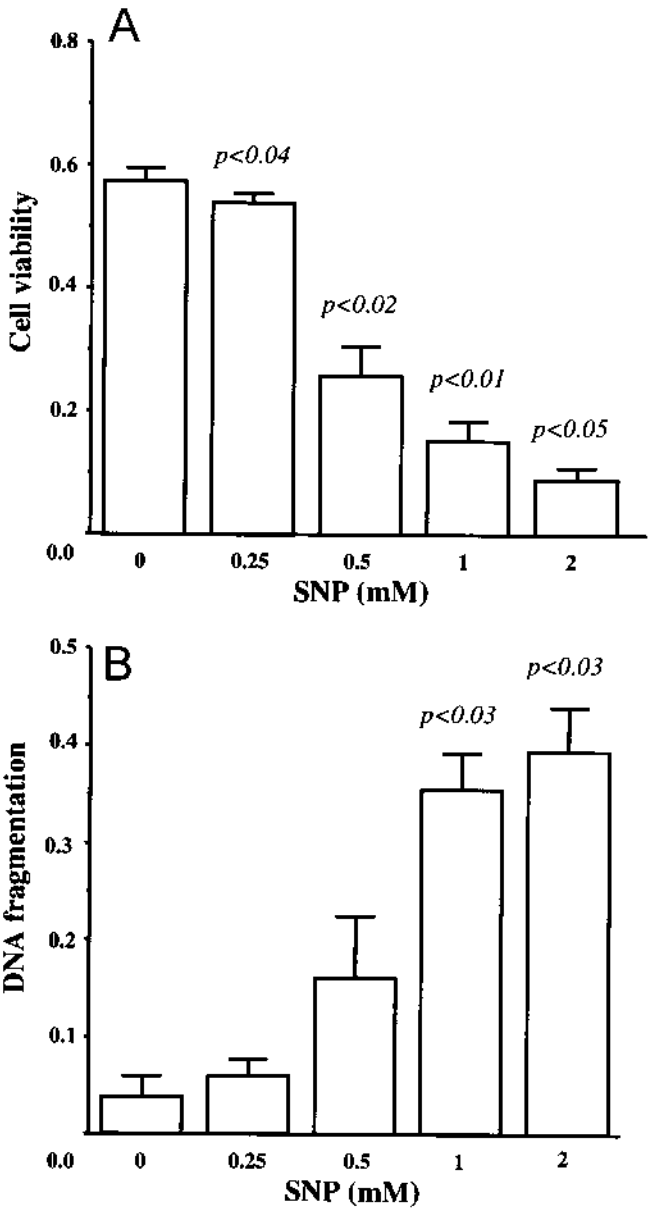

Figure 1. Dose dependent effects of sodium nitroprusside (SNP) on (A) cell viability (n = 3) and (B) DNA fragmentation (n = 3) in human OA synoviocytes. Cells were cultured in medium with or without various concentrations of SNP (0–2 mM) for 18 h. Mean ± SEM; p values vs control, Student t test.

Because of the ability of NO to induce apoptosis in synoviocytes[17,18], we examined the role of caspase-3 and caspase-9 in SNP induced synoviocyte death. Both caspase-3 (Z-DEVD-FMK) and caspase-9 (Z-LEHD-FMK) inhibitors at high concentrations were found to protect synoviocyte viability very effectively ($p < 0.04$ and $p < 0.05$, respectively) (Figure 3B).

Tyrosine kinases are the major activation pathway in NO induced synoviocyte death. The signaling cascade involved in NO induced human OA synoviocyte death was examined

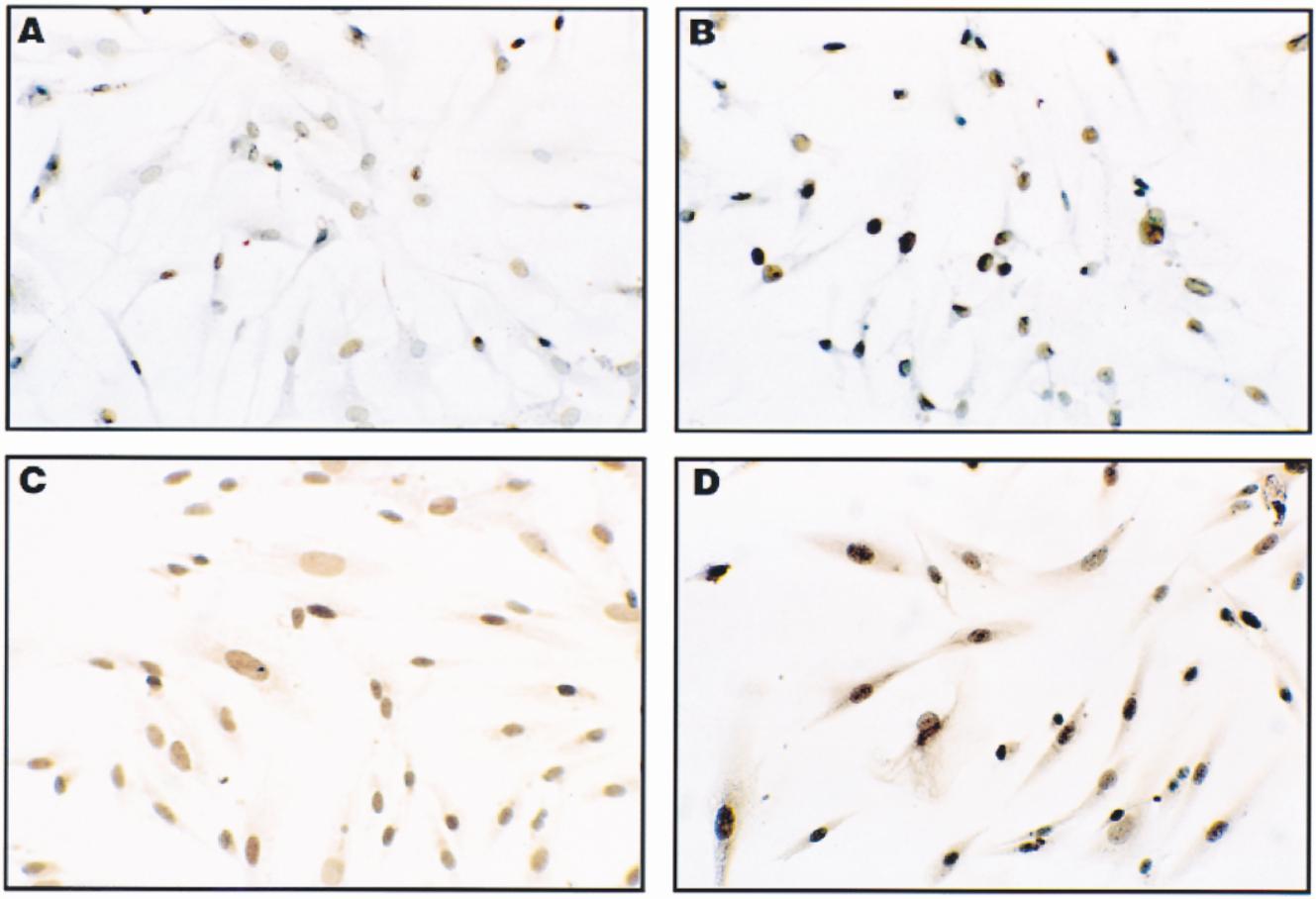

Figure 2. Effects of sodium nitroprusside (SNP) on synovial fibroblasts on DNA fragmentation (n = 3) (TUNEL reaction) (A, B); and on active caspase-3 level (n = 4) (C, D). Cells were incubated in the absence (A, C) or presence (B, D) of 0.5 mM SNP for 18 h (original magnification ×250).

Table 1. The effect of genistein on caspase-3 and caspase-9 activity and on Bcl-2 level in SNP treated human OA synoviocytes. Data are mean ± SEM (n = 4). Individual synoviocytes were cultured for 18 h. Caspase-3 and caspase-9 activity and Bcl-2 level in adherent cells were assayed by Ac-LEHD-AFC and Z-DEVD-AFC cleavage and by ELISA, respectively.

	Caspase-3, U/mg Protein	Caspase-9, U/mg Protein	Bcl-2, U/mg Protein
Control	16.9 ± 2.1	18.8 ± 3.7	107.4 ± 8.3
Genistein	14.9 ± 0.7	31.9 ± 6.5	107.3 ± 6.2
SNP	99.9 ± 0.1 (p < 0.0003)	100.1 ± 0.2 (p< 0.007)	90.2 ± 9.6
Genistein + SNP	13.4 ± 1.5	27.6 ± 6.0	92.9 ± 14.7

P values vs control without inhibitor; Student t test.

using specific pharmacological inhibitors of different intracellular signaling pathways. The concentrations of inhibitors tested in these experiments were the highest that can be used and were found to be noncytotoxic under the experiment conditions. Although the inhibition of protein kinase A (PKA) by KT-5720 and protein kinase G (PKG) by KT-5926 exerted some protective effect on cell viability (68.5% and 65.5% of the viability of control cells), a statistically significant protective effect was found only with inhibition of protein kinase C (PKC) by CalC ($p < 0.02$), p38 inhibition by SB-202190 ($p < 0.01$), and ERK1/2 inhibition by PD-98059 ($p < 0.03$). In the presence of these inhibitors, the cell viability reached 76%, 83%, and 86% of the corresponding controls, respectively. Interestingly, the tyrosine kinase (TK) inhibitor genistein completely and dose dependently protected synoviocytes against NO induced cell death (Figure 4).

Because genistein blocked receptor-type (EGFR-type TK) as well as nonreceptor related TK p60v-scr, we investigated the participation of both types of TK in NO mediated cell death. The EGFR-kinase inhibitor BPIQ-II showed only a minimal protective effect at maximal concentration, while EGFR-kinase inhibitor AG-490 at maximal concentration (25 μM) significantly protected synoviocyte viability (76% of control; $p < 0.004$). The 2 TK inhibitors (genistein and AG-490) were found to markedly block SNP induced PGE_2 synthesis in synoviocytes (Figure 5). These results suggested that the protective effect of genistein is not exercised solely by COX-2 inhibition (Figure 5, insert), and that the suppression of PGE_2 synthesis alone does not seem sufficient to protect the synoviocytes against the toxic effect

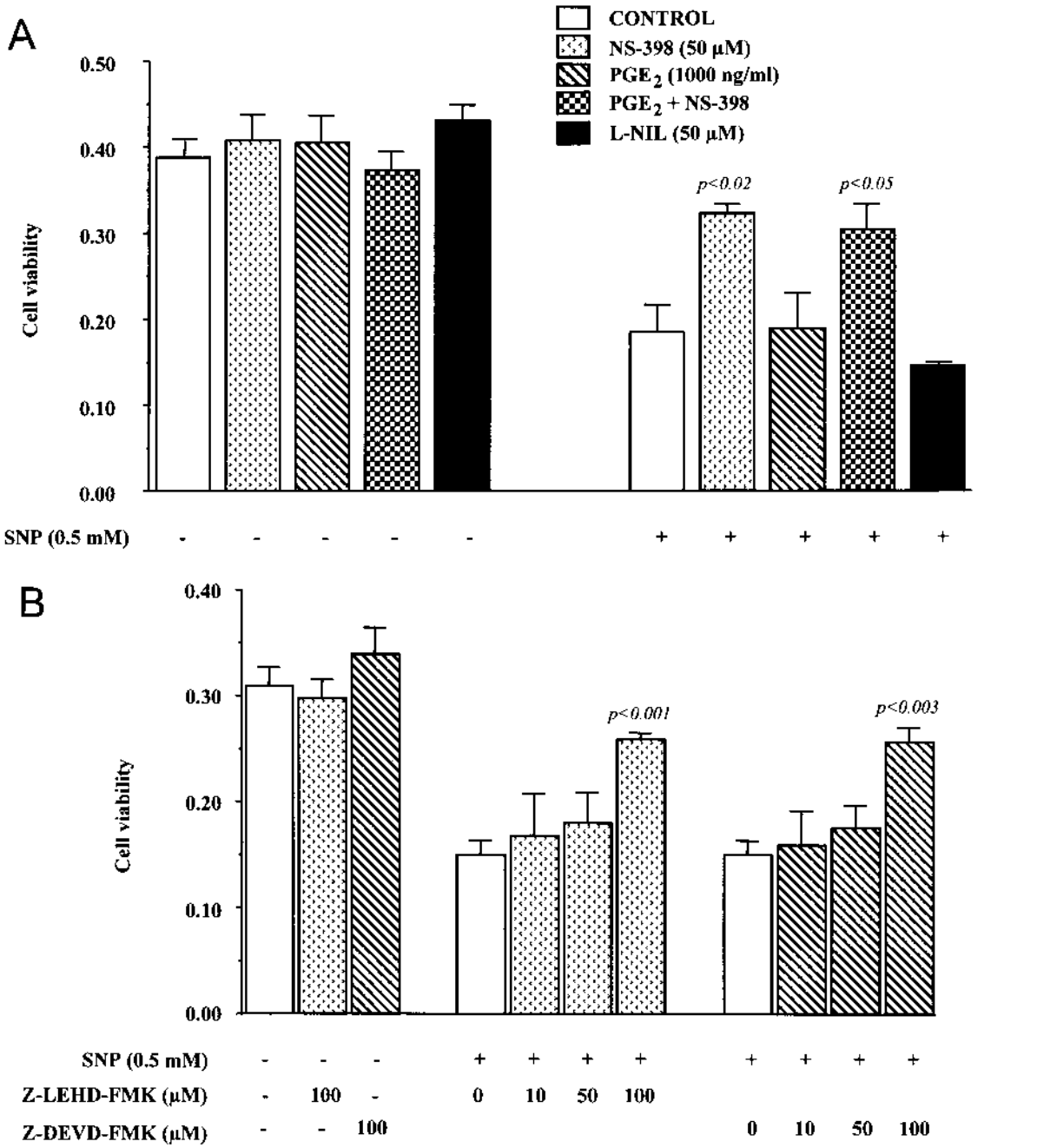

Figure 3. Effects of (A) COX-2 and iNOS inhibition and PGE_2 (n = 3) and (B) caspase-3 and caspase-9 inhibition (n = 3) on SNP treated human OA synoviocyte viability. Cells were preincubated 2 h with COX-2 inhibitor (NS-398) in the absence or presence of PGE_2, iNOS inhibitor (L-NIL), and with caspase-9 and caspase-3 inhibitors (Z-LEHD-FMK and Z-DEVD-FMK, respectively), followed by incubation with SNP (0.5 mM) for an additional 18 h. Mean ± SEM; p values vs control without each inhibitor, Student t test.

of NO. Other mechanisms are also likely implicated in that process; one possibility was the inhibition of caspase-3 and caspase-9 activity. We found that genistein completely inhibited both caspase-3 and caspase-9 activity in SNP treated synoviocytes (Table 1). However, AG-490 induced only a minor reduction in the activity of both caspases (data not shown).

SNP induced disruption of mitochondrial membrane potential ($\Delta\Psi_m$), reactive oxygen species formation, and cytochrome C release. Mitochondrial membrane potential was determined using the potential-dependent, aggregate-forming lipophilic cation JC-1. We found that NO induced a decrease of mitochondrial membrane potential in synoviocytes, shown by the incorporation of JC-1 monomers into the mitochondria (fluorescence in green, Figures 6C, 6D), compared to cytosolic J-aggregate formation at high

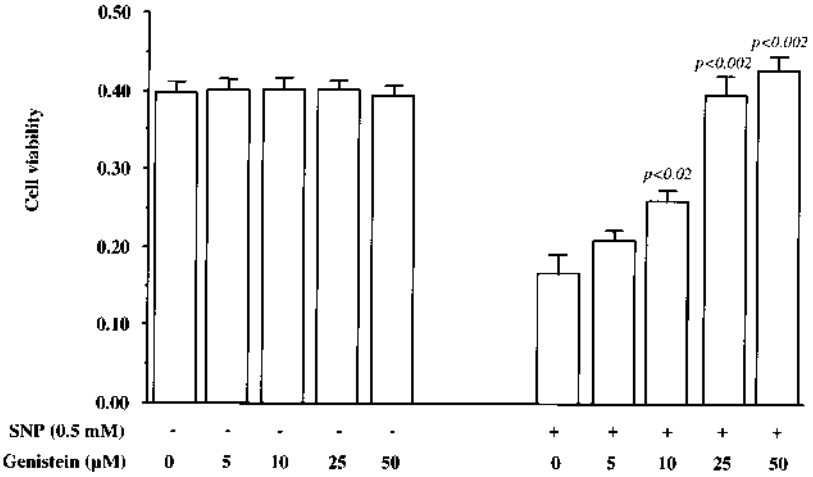

Figure 4. Effects of genistein on SNP induced cell death (n = 3) in human OA synoviocytes. Cells were preincubated with increasing concentrations of genistein for 2 h, followed by incubation with SNP (0.5 mM) for an additional 18 h. Mean ± SEM; p values vs SNP alone, Student t test.

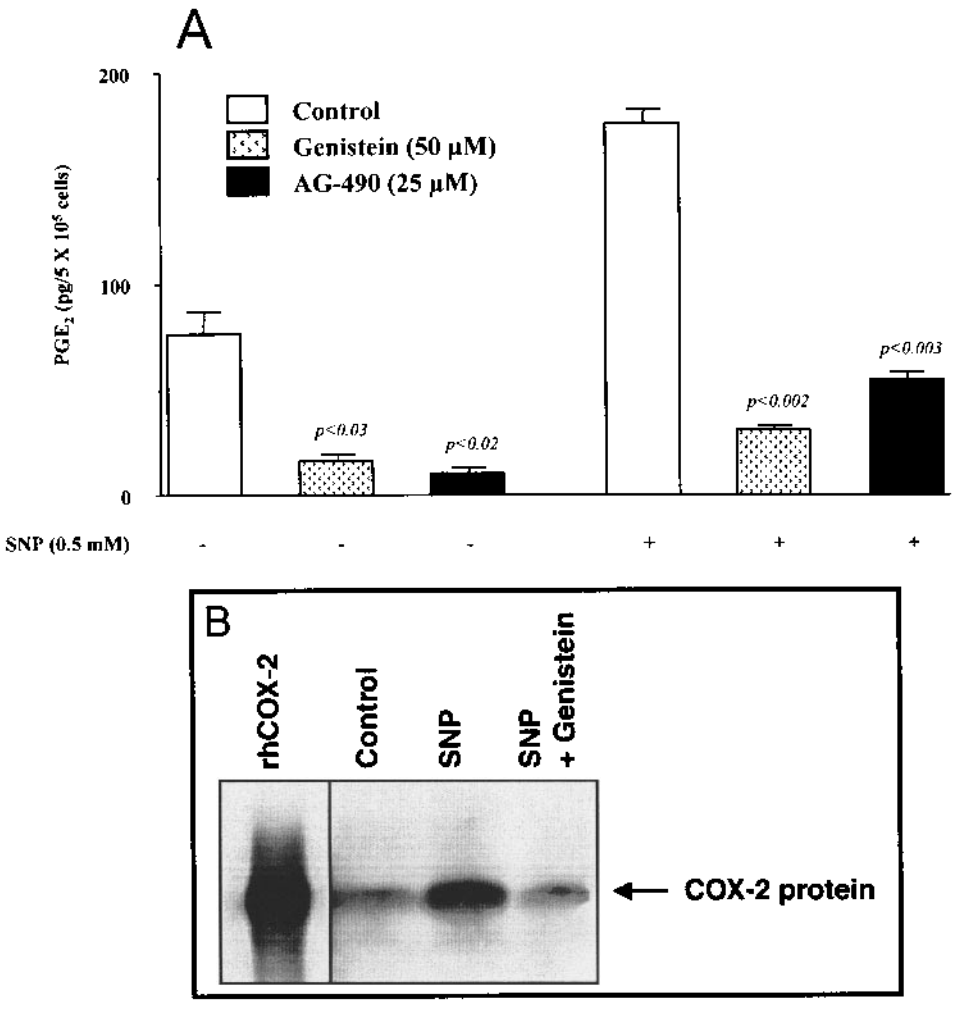

Figure 5. (A) Effects of tyrosine kinase inhibitors on PGE_2 secretion (n = 3). Cells were preincubated with AG-490 or genistein for 2 h, followed by incubation with SNP (0.5 mM) for an additional 18 h. Mean ± SEM; p values vs respective control, Student t test. (B) The effect of genistein on SNP induced COX-2 expression in OA synoviocytes (representative of 3 different experiments).

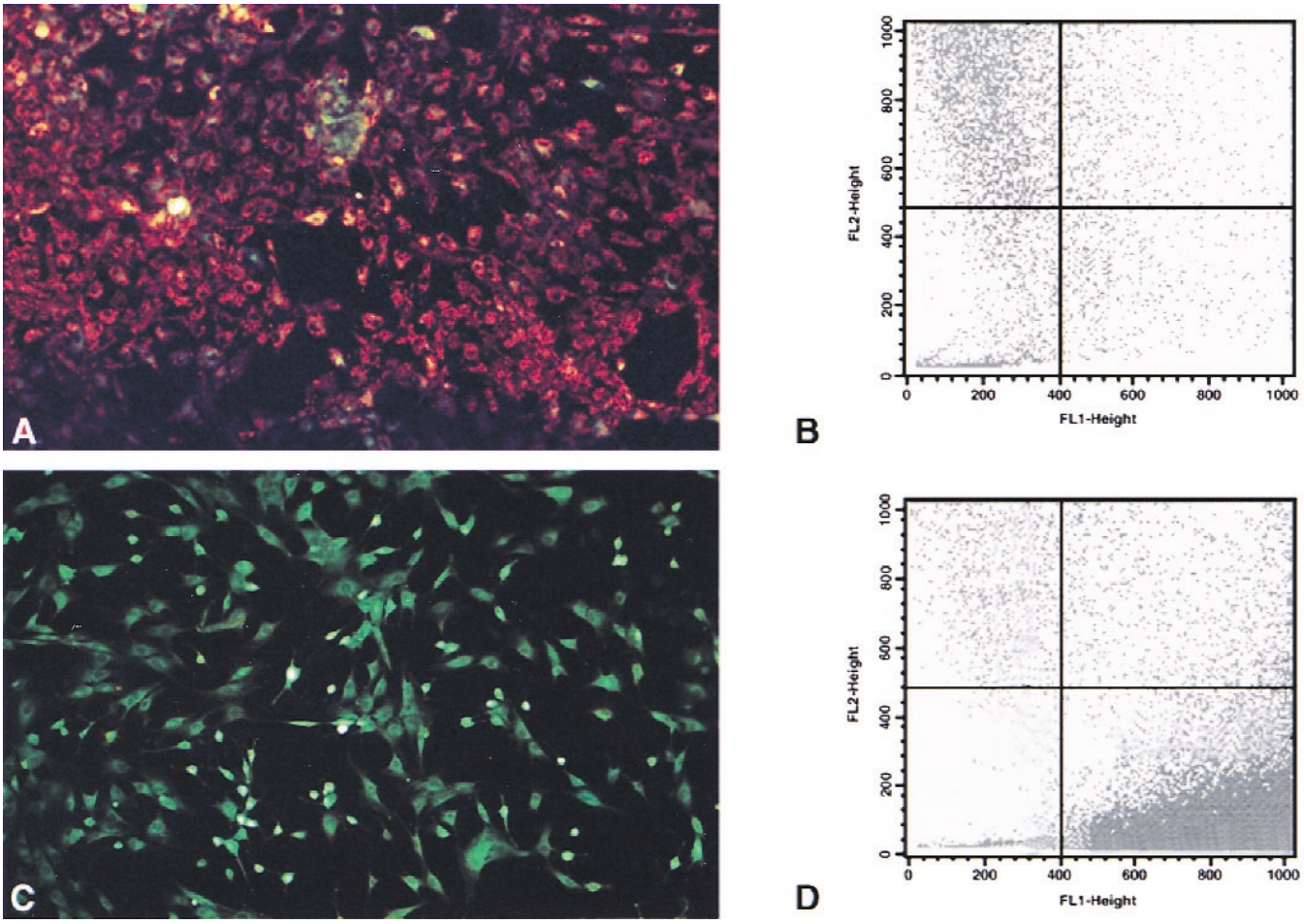

Figure 6. Effects of SNP on mitochondrial membrane potential of synovial cells (n = 4). Control (A, B) and SNP treated cells (0.5 mM, 18 h) (C, D) were incubated 10 min with JC-1 cationic dye (0.3 μg/ml), washed, and analyzed by fluorescence microscopy (A, C) or by flow cytometry (B, D). In control cells at high membrane potential, a red fluorescence developed, while NO induced a decrease of membrane potential, shown by the green fluorescence.

membrane potential in control cells (fluorescence in red, Figures 6A, 6B). A decrease in the mitochondrial membrane potential (due to increased permeability transition) could release cytochrome C, which could then activate the caspase cascade[21,22]. We tested this hypothesis using cyclosporine A, which specifically blocks mitochondrial cytochrome C release. Cyclosporine A protected the synoviocytes in a dose dependent manner against the toxic effect of NO and significantly increased their viability (Figure 7A). These results suggested that the engagement and activation of the mitochondrial amplification loop is the major mechanism of SNP induced synoviocyte cytotoxicity.

The concentration of tumor suppressor product p53 has been shown to be increased by a high concentration of NO and likely contributed to the disruption of mitochondrial membrane potential[23,24]. The concentration of p53 in SNP stimulated cells (143.2 ± 15.0 pg/10^6 cells) was found to be increased compared to control cells (67.6 ± 6.7 pg/10^6 cells) ($p < 0.03$). In contrast, Bcl-2, an endogenous inhibitor of mitochondrial permeability, was slightly decreased by SNP (Table 1).

Next, we investigated whether SNP induced cell death was due to a direct cytotoxic effect of NO and/or a reaction with free radicals. For this we used the scavengers for reactive oxygen species: NO scavenger (carboxy-PTIO, 0–100 μM), peroxynitrite ($ONOO^-$) scavenger (uric acid, 0–2 mM), and O_2^--H_2O_2 scavenger (taxifolin, 0–200 mM). We found that neither NO nor peroxynitrite ($ONOO^-$) alone was responsible for SNP induced cell toxicity (Figure 7B). From these experiments, we found that the decreased cell viability was related mainly to O_2^- and/or H_2O_2 formation as taxifolin significantly reduced synoviocyte cell death.

DISCUSSION

We found that NO induced synoviocyte death through COX-2 expression and PGE_2 synthesis. These results parallel the studies of von Knethen and Brune[25] and those of Hughes, *et al*[26], which described the induction of COX-2

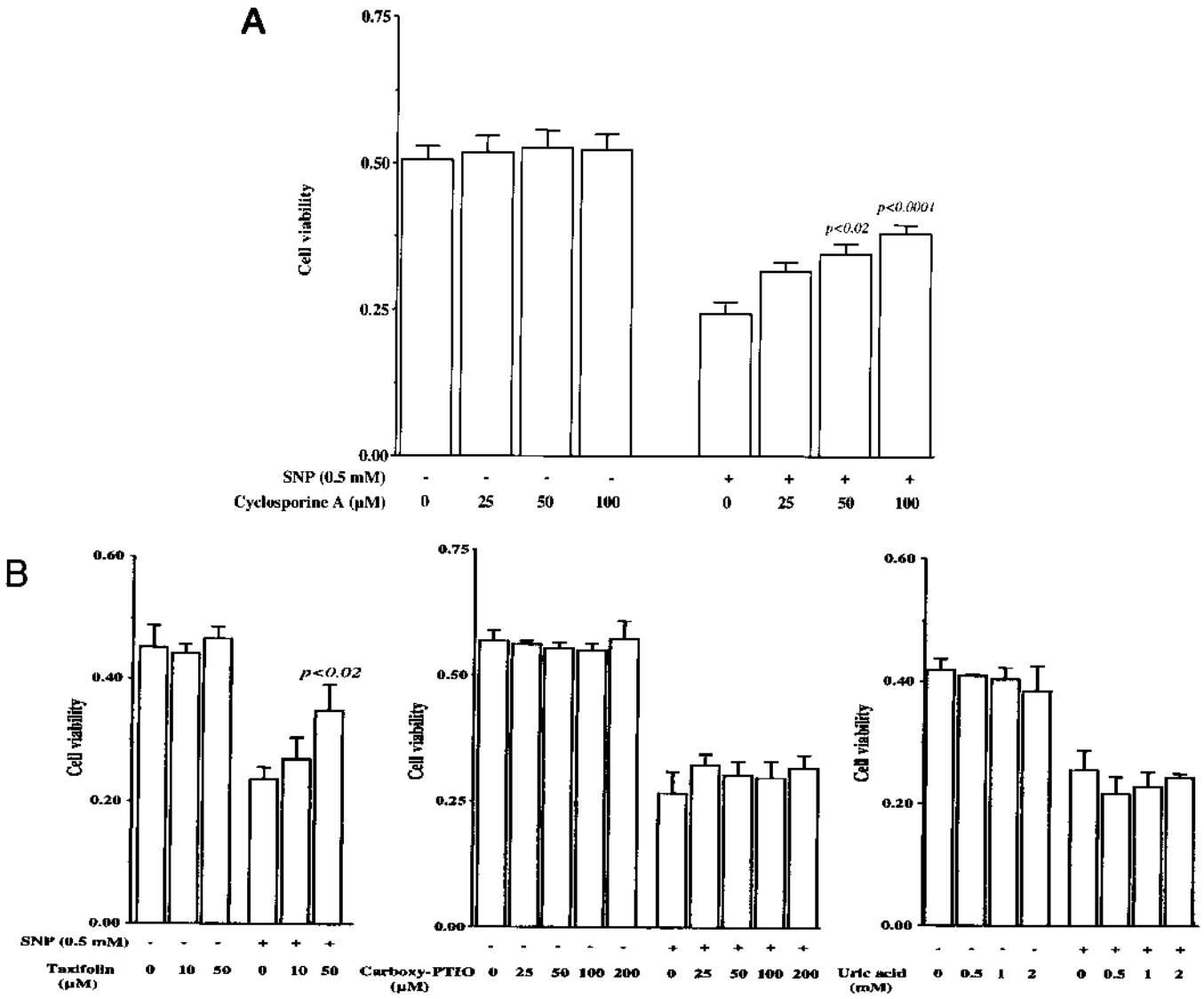

Figure 7. Effects of (A) inhibition of cytochrome C release (n = 3) and (B) ROS scavengers (n = 3) on SNP induced OA synoviocyte death. Cells were preincubated 2 h with (A) cyclosporine A or (B) taxifolin, carboxy-PTIO, or uric acid, followed by incubation with 0.5 mM SNP for an additional 18 h. Mean ± SEM; p values vs control without each inhibitor, Student t test.

protein by NO donors in macrophages and osteoblasts, respectively. We found that NO produced a cytotoxic effect on the synovial cells only in the presence of endogenously synthesized PGE_2, as NS-398, a specific COX-2 inhibitor, significantly improved the viability of SNP treated synoviocytes even in the presence of exogenous PGE_2. This implied that COX-2 expression was a major transducing pathway in regulating cellular susceptibility to NO in synoviocytes, a finding similar to that reported in RAW 264.7 macrophages[25]. The results are also closely in agreement with our report on human OA chondrocytes[14]. Our results also demonstrated that PGE_2 alone did not induce synoviocyte death, and therefore the exact mechanisms by which PGE_2 sensitized human OA synoviocytes to cell death are still not clear.

PGE_2 is reported to be an antiproliferative molecule and one of the inducers of apoptotic change in various types of cells. Increased PGE_2 was closely related to the induction of apoptosis in growth plate chondrocytes[27]. On the other hand, overexpression of COX-2 in several pathological conditions, such as colon carcinoma, points to the causative role of COX-2 in tumor initiation and/or promotion[28,29]. Thus, the effects of COX-2 and related PGE_2 in the regulation of apoptosis could very well be cell type-specific.

It is currently assumed that the activation of a cascade of cytoplasmic cysteine proteases (caspases) is essential for

apoptosis regardless of the initial death signal[30]. We observed that SNP generated NO caused synoviocyte death through activation of caspase-3 and caspase-9. Caspase-3 activation is considered a convenient marker of apoptosis and is regarded as the point of no return in the proapoptotic signaling cascade[31]. Inhibition of caspase-3 or caspase-9 almost completely blocked the SNP induced synoviocyte death, suggesting that under experimental conditions, cell death depends on the activity of caspases, which are largely absent in necrotic cells[32]. The recent report of Relic, *et al*[18], showing the presence of DNA fragmented into high molecular weight fragments, usually ranging from 30 kbp to 1 Mbp in human SNP treated synoviocytes, clearly indicated that synoviocyte death under these specific experimental conditions was related mainly to apoptosis. Our experiments also showed that the inhibition of mitochondrial function could be the major acting principle of NO induced synoviocyte death. Our finding of a significant change in mitochondrial membrane potential associated with the release of cytochrome C into the cytoplasm in SNP treated synoviocytes is strong evidence that the death of synoviocytes was related to apoptosis[33].

NO donors have the capacity to cause phosphorylation of various targets, including the mitogen activated protein kinase (MAPK) family[34,35]. We demonstrate that SNP can induce the activation of at least 2 MAPK, ERK1/2 and p38, as well as PKC. Specific inhibition of all 3 kinases significantly abolished SNP induced synoviocyte death, indicating that these kinases are mediators of this process. These results are in accord with the study associating p38 activation with apoptosis[36], but differ from the report that activation of ERK1/2 seemed to signal cell protection[37]. In the experiments, we decided to examine closely which signaling pathways mediated synovial cell death/apoptosis; we investigated the potential role of TK phosphorylation in the process. We found that genistein, a general TK inhibitor, completely inhibited SNP induced synoviocyte death. This protection was associated with complete inhibition of caspase-3 and caspase-9 activity as well as inhibition of COX-2 and PGE_2 secretion. Blanco, *et al*[38] also suggested that protein tyrosine phosphorylation plays a role in the regulation of COX-2 expression in endothelial cells. Genistein protected synoviocytes against NO induced cell death. Other TK inhibitors, such as AG-490, also completely abrogated NO induced PGE_2 secretion, however, with a much less pronounced protective effect on synoviocyte viability compared to genistein. These differences were found to be due to the significantly lower capacity of AG-490 to inhibit the increase in caspase-3 and caspase-9 activity. These findings again clearly showed that SNP induced synoviocyte apoptosis is not solely related to the induction of PGE_2 secretion.

This finding concerning the effect of genistein, which is a phytoestrogen, is very interesting. There have been a number of reports indicating that estrogen-type agents may protect against the development of clinical knee OA[39,40]. It is thus possible to speculate that the protective effect of the estrogen agents may work through the inhibition of chondrocyte apoptosis[41-43].

Although the exact mechanisms of NO mediated cytotoxity are still controversial, several possible systems have been suggested to explain this phenomenon. Nguyen, *et al*[44] demonstrated that NO could directly deaminate purine and pyrimidine bases in DNA and increase DNA breaks in human cells. As well, in rat insulinoma cells, it has been reported that NO could induce mitochondrial damage associated secondarily with DNA fragmentation and cell death[45]. NO could also react with O_2^-, yielding $ONOO^-$, a very potent oxidant that produces tyrosine nitration, which could interfere with the intracellular signaling pathways dependent on tyrosine phosphorylation[46]. Generation of $ONOO^-$ has also been reported to induce apoptosis in HL-60 cells[47]. We found that the scavengers for NO as well as for $ONOO^-$ had no protective effect on SNP induced synoviocyte cell death. However, significant protection was found with O_2^- and H_2O_2 scavenger. These findings are in agreement with the results of Terwel, *et al*[48], who showed that NO caused apoptosis in CHP212 cells by the production of H_2O_2 rather than NO or $ONOO^-$. Our results also support the recent finding of Del Carlo, *et al*[49], who demonstrated that NO mediated chondrocyte cell death by apoptosis requires the generation of additional reactive oxygen species.

Treatment with SNP was also found to increase cytoplasmic concentration of p53 protein. Interestingly, OA cultured synovial fibroblasts were already able to express a prominent level of p53 protein before the addition of SNP. This protein is known to mediate cellular response to DNA damage and maintain genomic stability[50], although in the presence of extensive DNA damage, p53 can direct cells toward apoptosis[51]. While it remains unclear why p53 was initially expressed in the cytoplasm of OA synoviocytes, these results are consistent with the report of Firestein, *et al*[52], that OA cultured synovial cells expressed abundant immunoreactive p53. The increase in the intercellular concentration of p53 could have greatly contributed to the release of cytochrome C and induction of apoptosis[25]. SNP treatment was also found to induce a decrease (20%) in the Bcl-2 concentration. This may also have contributed to the induction of apoptosis, since Bcl-2 can prevent cells from initiating apoptosis in response to a number of stimuli, including NO[53]. The antiapoptotic effect of Bcl-2, among others, was found to be mediated by the normalization of mitochondrial function.

We show that NO donor induces apoptosis in OA synoviocytes and provide evidence for a proapoptotic function of COX-2 and tyrosine kinases in these cells. As well, modulation of ERK1/2, p38 MAPK, and PKC activity correlated with the occurrence of NO induced cell death.

Moreover, the generation of O_2^{-}-H_2O_2 was associated with the disruption of mitochondrial membrane potential, and with the release of cytochrome C, these were the major constituents of SNP mediated apoptotic death in OA synoviocytes. These results highlight the potential regulating role of NO in the pathophysiology of OA.

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