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Stimulation of cyclooxygenase-2-activity by nitric oxide-derived species in rat chondrocyte: lack of contribution to loss of cartilage anabolism

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Abstract

Cross-talk between inducible nitric oxide synthase (NOS II) and cyclooxygenase-2 (COX-2) was investigated in rat chondrocytes. In monolayers, interleukin-1β (IL-1β) induced COX-2 and NOS II expression in a dose- and time-dependent manner, to produce high prostaglandin E_2 (PGE_2) and nitrite (NO_2^-) levels in an apparently coordinated fashion. COX-2 mRNA was induced earlier (30 min. *versus* 4 hr) and less markedly (4-fold *versus* 12-fold at 24 hr) than NOS II, and was poorly affected by the translational inhibitor cycloheximide (CHX). IL-1β did not stabilize COX-2 mRNA in contrast to CHX. Indomethacin and NS-398 lacked any effect on NO_2^- levels whereas L-NMMA and SMT reduced PGE_2 levels at concentration inhibiting NO_2^- production from 50 to 90%, even when added at a time allowing a complete expression of both enzymes (8 hr). Basal COX activity was unaffected by NO donors. The SOD mimetic, CuDips inhibited COX-2 activity by more than 75% whereas catalase did not. Inhibition of COX-2 by CuDips was not sensitive to catalase, consistent with a superoxide-mediated effect. In tridimensional culture, IL-1β inhibited radiolabelled sodium sulphate incorporation while stimulating COX-2 and NOS II activities. Cartilage injury was corrected by L-NMMA or CuDips but not by NSAIDs, consistent with a peroxynitrite-mediated effect. These results show that in chondrocytes: (i) *COX2* and *NOS II* genes are induced sequentially and distinctly by IL-1β; (ii) COX-1 and COX-2 activity are affected differently by NO-derived species; (iii) peroxynitrite accounts likely for stimulation of COX-2 activity and inhibition of proteoglycan synthesis induced by IL-1β.

Keywords: Chondrocytes; Interleukin-1β; Peroxynitrite; Cyclooxygenase-2; Non-steroidal anti-inflammatory drugs; Nitric oxide synthase inhibitors

1. Introduction

Prostaglandin endoperoxide H synthase (PGHS) or cyclooxygenase (EC 1.14.99.1) is a bifunctional enzyme with distinct cyclooxygenase and hydroperoxidase activities. COX exists in at least two isoforms with quite similar molecular weights (~70 KDa) and subtle differences in their catalytic activity. COX-1 is expressed constitutively and was first characterised, purified and cloned from sheep vesicular glands. In contrast, COX-2 is expressed in few tissues under basal conditions [1]. However, it is induced in various cell types including chondrocytes when exposed to mitogens, bacterial endotoxins or pro-inflammatory cytokines as IL-1 [2,3]. Besides, the free radical NO has emerged as an important signal and effector molecule in mammalian physiological processes including neurotransmission, vasodilatation and inflammation. NO is synthesised from the guanidino nitrogen group of L-Arg by the catalytic reaction of NOS (EC 1.14.13.39) isoforms [4]. The constitutive forms produce small amounts of NO and were found primarily to be expressed in endothelial (eNOS, NOS III) and neuronal (nNOS, NOS I) cells. An inducible form (iNOS, NOS II), producing large quantities of NO for a long period of time, has been described in many cell types in-

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Abbreviations: ACT D, actinomycine D; CHX, cycloheximide; COX, cyclooxygenase; CuDips, Cu(II) $(3,5\text{-diisopropylsalicylate})_2$; DMSO, dimethyl sulfoxide; D-NMMA, N^{ω}-monomethyl-D-arginine; FCS, heat-inactivated fetal calf serum; H_2O_2, hydrogen peroxide; IL-1β, interleukin-1β; L-Arg, L-arginine; L-NMMA, N^{ω}-monomethyl-L-arginine; NO, Nitric oxide; NO_2^-, nitrites; NOS, nitric oxide synthase; NSAID, non steroidal anti-inflammatory drug; PGE_2, prostaglandin E_2; RT-PCR, reverse transcription-polymerase chain reaction; SIN-1, 3-morpholinosydnonimine; SMT, S-methylisothiourea; SOD, superoxide dismutase.

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cluding chondrocytes [5] after stimulation by inflammatory agents. Therefore, both families of enzymes share in common to possess constitutive isoforms, namely COX-1 and cNOS (NOS I and III), which are responsible for basal production of mediators in physiological conditions, and inducible ones, namely COX-2 and iNOS (NOS II), which are overexpressed upon stimulation with proinflammatory stimulus and may play a pathological role. As a consequence, in many inflammatory disorders, both COX and NOS pathways are activated to various degree and it is questionable as to whether these pathways may be functionally linked or not [6].

During rheumatoid arthritis (RA) and osteoarthritis (OA), IL-1 is thought to play a pivotal role in cartilage damage due to its duality to suppress the synthesis of specific extracellular matrix molecules and to promote the imbalance between excessive cartilage destruction and defective cartilage repair [7]. Additionally, high levels of PGE_2 and NO-end products have been reported in synovial fluid of patients with joint diseases as a result of COX-2 and NOS II expression within synovium [8] and cartilage [9]. Prostaglandins and NO are both able to affect chondrocyte metabolism. Thus, prostaglandins may decrease chondrocyte proliferation [10], modulate metalloprotease activity [11] and inhibit aggrecan synthesis [12] in chondrocytes. Otherwise, NO is now considered as an important effector of IL-1 in cartilage and is thought to be involved, at least in part, in induction of apoptosis, decrease of IL-1Ra production and suppression of proteoglycan synthesis [13]. Failure to induce arthritis in NOS II knock-out mice [14] and cartilage protection provided by NOS inhibitors in the IL-1 arthritis model in rat [15] further argue for a crucial role of inducible NOS pathway in cartilage damage. However, cartilage protection provided by NOS inhibitors may result from a modulation of prostaglandins production additionnally to the reduction of NO synthesis. Indeed, a significant decrease in PGE_2 levels was reported in the synovial fluid of arthritic [16] or osteoarthritic [17] animals treated orally with NOS inhibitors and this was accompanied by a general reduction of catabolic factors levels within joint tissues [18].

In light of these experimental data, it has to be clarified if NO modulates directly chondrocyte metabolism or whether it may act through the modulation of PGE_2 production. Various *in vitro* [19,20] and *in vivo* [19,21,22] studies have underlined that the cross-talk between NOS and COX pathways depends both on the tissue and inducing agent and cannot be extrapolated from a cell type to another one. Moreover, the partial correcting effect of NOS inhibitors in the IL-1 arthritis model in rat [15] suggests that mediators other than NO itself may participate in the inhibition of proteoglycan synthesis. In the present study, we investigated therefore any possible cross-talk between COX-2 and NOS II in chondrocyte and the relevance of the modulation of COX-2 in the inhibitory effect of IL-1 on proteoglycan synthesis. We characterised firstly the kinetics of COX-2 and NOS II expression activity under stimulation with range doses of IL-1. Secondly, we investigated the interplay between COX-2 and NOS II pathways using inhibitors with variable selectivities for inducible isoforms: L-NMMA and SMT as non selective and selective NOS II inhibitors, respectively, indomethacin and NS-398 as non-selective and selective COX-2 inhibitors, respectively. Finally, we investigated whether NO-derived products affected COX-2 activity and participated in the inhibitory effect of IL-1 on proteoglycan synthesis, using CuDips or catalase as scavengers.

2. Materials and methods

2.1. Materials

All culture medium and reagents for molecular biology were obtained from Life technologies. ACT D, alginate, Bicinchoninic acid assay kit (BCA-1), Bovine liver catalase, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT), CHX, CuDips, DMSO, ethidium bromide, indomethacin, L-Arg, D-NMMA, L-NMMA, N-naphtylethylenediamine dihydrochloride, SIN-1, SMT, SNAP, sulfanilamide and trypsin were purchased from Sigma-Aldrich Chemical Co. Collagenase was obtained from Boehringer Mannheim. NS-398 was purchased from Tebu. Anti-type II collagen goat antibody and Fluorescein isothiocyanate (FITC) conjugate donkey anti-goat or goat anti-rabbit immunoglobulin G (IgG) were obtained from Jackson Immunochemicals. Lactate deshydrogenase (LDH) kit (LK-100) and goat polyclonal anti-C-terminus COX-2 antibody were purchased from Oxford Biomedical Research Inc. Rabbit polyclonal anti-iNOS antibody was obtained from Calbiochem. Bovine serum albumin (BSA), PVDF (polyvinylidene fluoride) membranes and MetaPhor agarose gel were purchased from Interchim, Millipore and FMC Bioproducts respectively. $Na_2{}^{35}SO_4$ was obtained from Amersham. Soluene 350, Ultima gold were purchased from Packard. Recombinant human IL-1β was a generous gift from Rhône-Poulenc Rorer.

2.2. Isolation and culture of chondrocytes

A pool of articular chondrocytes was obtained from knee and hip joints of healthy male Wistar rats (180–200 g) (Charles River). Briefly, rats were killed under dissociative anaesthesia, their joints were quickly dissected under laminar flow and cartilage pieces were taken with a scalpel from both patellae, tibial plateaus, femoral condyles and femoral heads. Chondrocytes were further isolated by sequential digestion with trypsin (0.2%, w/v solution) and collagenase (0.2%, w/v solution) according to the method of Green [23]. Cells were initially plated in DMEM/Ham's F12 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 50 μg/mL gentamicin, 0.25 μg/mL amphotericin B. Cells were cultured at confluence at 37° in a humidified atmosphere

containing 5% CO_2 and were used at passage 2 or 3. Chondrocyte phenotype was controlled by ability to produce type II collagen as determined by immunofluorescence study with anti-type II collagen goat antibody and FITC conjugate. In all subsequent experiments, chondrocytes were cultured under low FCS conditions (2.5%, v/v) to prevent COX-2 induction by growth factors and were used either in monolayers or as tridimensional cultures in alginate beads, for measurement of proteoglycan synthesis. Briefly, cells were suspended in sterile filtered low viscosity alginate solution (1.2%, w/v) at a concentration of 6×10^6 cells/mL and slowly expressed through a 22 gauge needle into a 102 mM $CaCl_2$ solution. After instantaneous gelation, beads were allowed to further their polymerisation for a 10 min. period in $CaCl_2$ solution. After three washes in 0.15 M NaCl, beads were maintained in complete culture medium for 6 days before use.

2.3. Effect of IL-1β on COX-2 and NOS II expression

Chondrocytes were seeded in 24-well plates at a density of 2.5×10^5 cells per well and cultured overnight under low FCS conditions before IL-1 challenge. In the dose-response experiments, cells were incubated 24 hr with or without recombinant human IL-1β in the range of 0.025 to 250 U/mL. In one experiment, culture supernatants were removed at the end of incubation and were assayed for PGE_2 and nitrites as described below. Cell layers were lysed with a 0.3 M NaOH solution containing 0.5% (w/v) of SDS and were further analysed for total protein content using the BCA-1 assay kit. In another experiment, cell layers were washed with ice-cold phosphate-buffered saline pH 7.4 (PBS) at the end of incubation, scrapped and lysed by sonication in 500 μL of ultrapure water, then subjected to western-blotting as described below.

In our time-response experiments, chondrocytes were stimulated with 25 U/mL IL-1β for either 0.5, 1, 2, 4, 6, or 24 hr. For each time of incubation, supernatants were removed and assayed for PGE_2 and nitrites levels. In some experiments, in order to distinguish whether IL-1 may regulate NOS II and COX-2 in a different way, cells were either pre-treated with 10 μg/mL CHX 2 hr before IL-1β stimulation or post-treated with 5 μg/mL ACT D 1 hr after IL-1β. All chondrocytes layers were washed twice with ice-cold PBS and subjected to total RNA extraction by the guanidium thiocyanate-phenol-chloroform method [24] using the Trizol solution. Total RNA was quantitated by spectrophotometry at 260 nm. Approximately 2.5 μg total RNA was recovered per 2.5×10^5 cells, whatever treatment.

2.4. Study of NOS/COX interplay

Interplay between inducible NOS and COX pathways was investigated in chondrocytes treated for 24 hr with IL-1β (25 or 250 U/mL). Indomethacin or NS-398, as COX inhibitors, or L-NMMA and SMT, as NOS inhibitors, were added to culture medium at the same time as IL-1β. Compounds were used at concentrations ranging from 1 μM to 1 mM except SMT which was used at 0.1 and 1 mM. NSAIDs were dissolved in DMSO and added to culture medium in such a way that final concentration of DMSO never exceeded 0.5% (v/v). In these experiments, cytotoxicity was checked out by measuring LDH activity in culture supernatants with a commercially available colorimetric assay kit (LK-100) and indomethacin (1 mM) was abandoned due to cytotoxicity (3- to 5-fold increase). Inhibitors alone were used as controls. At the end of incubation, culture supernatants were removed and assessed for nitrite (NO_2^-) and PGE_2 levels as described below. The specificity of L-NMMA effects towards NO pathway was confirmed with D-NMMA and by studying the reversibility of NOS inhibition by an excess of L-Arg. Briefly, chondrocytes stimulated for 24 hr with IL-1β (25 or 250 U/mL) were treated with either D-NMMA (0.1 and 1 mM) or L-NMMA (0.1 or 1 mM) in the presence of L-Arg (5 mM).

To assess the contribution of NO radical species to the modulation of COX pathway, chondrocytes were stimulated for 24 hr with IL-1β (25 U/mL) in the presence of CuDips at 10 or 1 μM or catalase at 500 U/mL. As the modulation of COX-2 pathway by NOS inhibitors or radical scavengers can be either at the level of protein expression or enzyme activity, the effects of L-NMMA (1 mM), SMT (0.1 mM), CuDips (10 μM) or catalase (500 U/mL) were also studied when they were added 8 hr after IL-1β (25 U/mL). In all cases, supernatants were monitored for NO_2^- and PGE_2 as described below.

The effect of exogenous NO on basal COX activity was studied using the NO donors SNAP and SIN-1. Briefly, unstimulated chondrocytes were cultured for 24 hr in the presence of 1 mM of SNAP or 0.1 mM of SIN-1 and both nitrites and PGE_2 levels were assessed in culture supernatants.

2.5. Western-Blotting for NOS II and COX-2 expression

Chondrocytes lysates were boiled in a ratio of 1:1 with gel loading buffer (125 mM Tris HCl, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol and 0.004% v/v bromophenol blue). Electrophoresis was performed by using 10% (w/v) SDS-polyacrylamide gel (50 μg protein per lane) according to Laemmli [25]. Separated proteins were transferred onto PVDF membranes (Immobilon-P) and non-specific IgGs were blocked with 3% (w/v) BSA in TBST (Tris buffered saline) solution (9 mM Tris-HCl, pH 9.0, 154 mM NaCl, 0.05% v/v Tween 20). Membranes were incubated for 1 hr with primary antibodies, *i.e.* goat polyclonal anti-C-terminus COX-2 antibody (1:100) or rabbit polyclonal anti-iNOS antibody (1:100). After 6 washes with TBST solution, membranes were further incubated for 1 hr with secondary antibodies conjugated to alkaline phosphatase (1:20000). Finally, membranes were washed again with

Table 1
Primers used for reverse transcription and PCR amplification

Primer sequence	Localisation on cDNA	Intended size
COX-1		
F 5′GGA-GTC-TCT-CGC-TCC-AGT-TTC-C3′	11–32	
R 5′TCT-ACC-CCA-TGG-CCT-AAG-GCC-T3′	671–692	682 bp
COX-2		
F 5′TTC-CGA-GCT-GTG-CTG-CTC-TGC-GC3′	131–153	
R 5′AGT-CCT-CGG-GTG-AAC-CCA-GGT-CC3′	731–753	623 bp
NOS II		
F 5′CTG-GGA-GAA-AAC-CCC-AGG-TGC-T3′	101–122	
R 5′ATG-GCC-GAC-CTG-ATG-TTG-CCA-C3′	721–742	642 bp
G3PDH		
F 5′ACG-GAT-TTG-GCC-GTA-TTG-GCC-GC3′	49–72	
R 5′TGG-TCC-TCA-GTG-TAG-CCC-AGG-AT3′	840–863	815 bp
RDT 17		
5′GAC-TCG-AGT-CGA-CAA-GC$(T)_{17}$3′		—

F, forward; R, reverse.

TBST, incubated for 5 min. with BCIP/NBT and reaction was stopped by washing membranes in methanol.

2.6. Reverse transcription-PCR for NOS II and COX-2 expression

Integrity of RNA was analysed by subjecting 1 μg of total RNA to electrophoresis through a 1% (w/v) agarose gel containing 2 M formaldehyde. RT-PCR was performed as described previously [26]. Briefly, cDNA was prepared by reverse transcription of 2 μg total RNA in 50 μL of solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 2 mM dithiothreitol, 0.4 μM primer (RDT17, Table 1), 0.5 mM dNTPs, 0.2 U/μL RNase inhibitor and 4 U/μL SuperScript RNase H^- reverse transcriptase. Reaction mixtures were incubated at 42° for 2 hr. The reverse transcription products were diluted 10-fold, and used for each PCR amplification. PCRs were performed in 50 μL of solution containing 5 μL cDNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 μM of each primers (see sequences in table 1), 0.2 mM each dNTPs and 0.01 U/μL *Taq* polymerase. The samples were subjected to one cycle at 94° for 5 min., 60° or 57° for 1 min. and 72° for 40 min. to synthesise the second cDNA strand. Amplification was performed for 30 cycles at 94° for 1 min., 60° or 57° for 1 min. and 72° for 1 min. using a thermocycler (Techne). The final extension step lasted for 10 min. at 72°. RT-PCR products were electrophorised in a 1.8% (w/v) MetaPhor agarose gel containing 0.2 μg/mL ethidium bromide. Primers were designed by computer analysis to be 100% homologous with sequences of COX-1, COX-2, NOS II, or G3PDH and to lack any cross linking with other known sequences recorded in data bank. Amplified cDNAs were sequenced by Genome Express SA and were shown to be identical to intended sequences (Genbank Database, accession numbers U18060 for COX-1, S67722 for COX-2, D14051 for NOS II and M17701 for G3PDH). RT-PCR were done twice in two separate experiments.

2.7. Contribution of COX-2 and NOS II-derived species to proteoglycan synthesis inhibition

In order to evaluate the respective contribution of COX-2 and NOS II in cartilage anabolism, chondrocytes encapsulated in alginate beads were incubated for 48 hr with IL-1β (25 U/mL/day) in the presence of indomethacin, NS-398, L-NMMA or CuDips. NSAIDs were used at 1 μM, whereas L-NMMA was used at 1 mM and CuDips at 10 μM. These concentrations were chosen because they were highly effective in modulating one or both of the inducible pathways. The medium was changed daily. At the end of incubation (48 hr), culture media were removed and stored at −80°C until NO_2^- or PGE_2 assays, whilst chondrocytes beads were further incubated in culture medium supplemented with 10 μCi/mL of $Na_2{}^{35}SO_4$ for 4 h at 37° in a 5% CO_2 atmosphere. Alginate beads were washed five times with 0.15 M NaCl and solubilised in citrate-EDTA buffer (55 mM/20 mM). Isolated chondrocytes were further digested overnight at 60° in papain buffer (0.2 M NaH_2PO_4, 0.01 M EDTA, 0.01 M cysteine and 6.75 U/mL papain). The [^{35}S]-proteoglycan content was measured by liquid scintillation counting (Ultima gold) with a LKB 1214 counter (Wallac).

2.8. Assays of NO_2^- and PGE_2

The stable end product NO_2^- was used as an index of NO production. NO_2^- levels were measured in culture supernatants by the Griess colorimetric assay [27]. Briefly, 100 μL of culture supernatant were mixed with 100 μL of Griess

reagent [sulfanilamide (1%, w/v) in 5% (v/v) of H_3PO_4 and *N*-Naphtylethylenediamine dihydrochloride (0.1% w/v) in H_2O] for 5 min. Optical density was read at 550 nm with a MR5000 (Dynatech, France) microplate reader and data were expressed as cumulated nitrite level over 24 or 48 hr by comparison with a standard curve of sodium nitrite (5 to 50 μM). Limit of detection was 1 μM.

PGE_2, the main metabolite of cyclooxygenase in chondrocytes [28], was measured in supernatants using a commercially available enzyme-linked immunosorbant assay (Assay Desing Inc.) according to the manufacturer's instructions. This assay showed no significant cross-reactivity with other prostanoids and sensitivity was around 30 pg/mL (manufacturer's instructions).

2.9. Statistical analysis

Data are expressed as mean ± SD. Each value is the mean of at least three samples. For nitrite or PGE_2 production, data are expressed as cumulated levels vs controls or as percentage of cumulated levels vs IL-1β controls. For proteoglycan synthesis, data are expressed as percentage of variation of radiolabelled sodium sulphate incorporation vs untreated controls. The results were analysed by ANOVA followed by a Fisher test. Differences with $P < 0.05$ were considered as significant.

3. Results

3.1. Expression of COX-2 and NOS II in response to IL-1

Rat articular chondrocytes exposed for 24 hr to IL-1β produced NO and prostaglandins in a dose-dependent manner, whereas their basal production of mediators was low. The stimulating effect of IL-1β was significant from 0.25 U/mL on both mediators although the dose-response curves showed somewhat different profiles, with NO_2^- being more responsive than PGE_2 to low IL-1 concentrations (Fig. 1A). Western-blot analysis failed to detect COX-2 and NOS II proteins under basal conditions but showed that both enzymes were dose-dependently expressed after IL-1 challenge (Fig. 1B). The stimulating effect of IL-1β was detected from 25 U/mL only, which may simply reflect the lowest sensitivity of western blotting compared to assays of mediators.

In the presence of 25 U/mL of IL-1β, the release of NO_2^- and PGE_2 showed an early lag phase until 6 hr and a secondary productive phase until 24 hr (Fig. 2). Interestingly, NO_2^- and PGE_2 evolved in an apparently coordinated fashion, although the maximal magnitude of variation was greater for prostaglandins (209-fold) than for nitrites (38-fold). Immunoreactivity for COX-2 was detected earlier than for NOS II but was maximal at 24 hr for both enzymes (data not shown).

3.2. Regulation of COX-2 and NOS II genes by IL-1

COX2 gene was constitutively expressed in rat chondrocytes, whereas *NOS II* gene failed to be consistently detected (Table 2). Surprisingly, a transient 2-fold increase in COX-2 mRNA was observed 30 min. after the changing of culture medium, although the latter contained a low FCS concentration (data not shown). NOS II did not show any significant changes in the same experimental conditions.

After IL-1 stimulation, steady-state levels of COX-2 mRNA increased from 30 min., peaked at 4 hr (approximately 4-fold increase), then remained stable until 24 h (Table 2). In contrast, *NOS II* gene was weakly expressed in the first hours following IL-1 challenge, became strongly detectable from 4 hr (approximately 12-fold increase), then remained elevated until 24 hr. *COX1* gene was expressed constitutively and did not change obviously after IL-1 stimulation (Table 2).

CHX alone induced COX-2 mRNA expression from 2 hr with a magnitude comparable to that obtained with IL-1β (Table 2). However, the stimulating effect of CHX was not as sustained as with IL-1 at 24 hr. In these conditions, *NOS II* gene expression was delayed when compared to *COX-2* and the magnitude of induction remained lower (4 to 6-fold) than with IL-1. Co-treatment of chondrocytes with CHX and IL-1β induced *COX2* and *NOS II* genes with distinct kinetics (Table 2). The stimulating effect of IL-1 on COX-2 mRNA was less affected by CHX than its effect on NOS II mRNA, except at 24 hr.

Post-treatment of chondrocytes with ACT D did not affect the basal expression of COX-1 and G3PDH whereas it abolished the stimulating effect of IL-1β on COX-2 and NOS II (data not shown). In addition, the basal and the IL-1 stimulated COX-2 mRNA levels decreased progressively after ACT D treatment ($T_{1/2} = 1$ hr), which was not the case for COX-1 and G3PDH. ACT D post-treatment suppressed also the stimulating effect of CHX on NOS II mRNA (data not shown) but did not affect its stimulating effect on COX-2 ($T_{1/2} > 4$ hr) (data not shown).

3.3. Interplay between COX and NOS pathways

In our experimental conditions, NS-398 and indomethacin reduced the production of PGE_2 induced by IL-1β (Fig. 3A) but not the basal level of PGE_2 and NO_2^- (data not shown). In contrast, NSAIDs failed to lower NO_2^- levels induced by of IL-1β (Fig. 3B), even at the highest concentration tested (0.1 mM for indomethacin, 1 mM for NS-398).

L-NMMA inhibited the low basal level of NO_2^- (62% inhibition at 1 mM) whereas SMT was ineffective (data not shown). Both compounds inhibited NO_2^- levels in the pres-

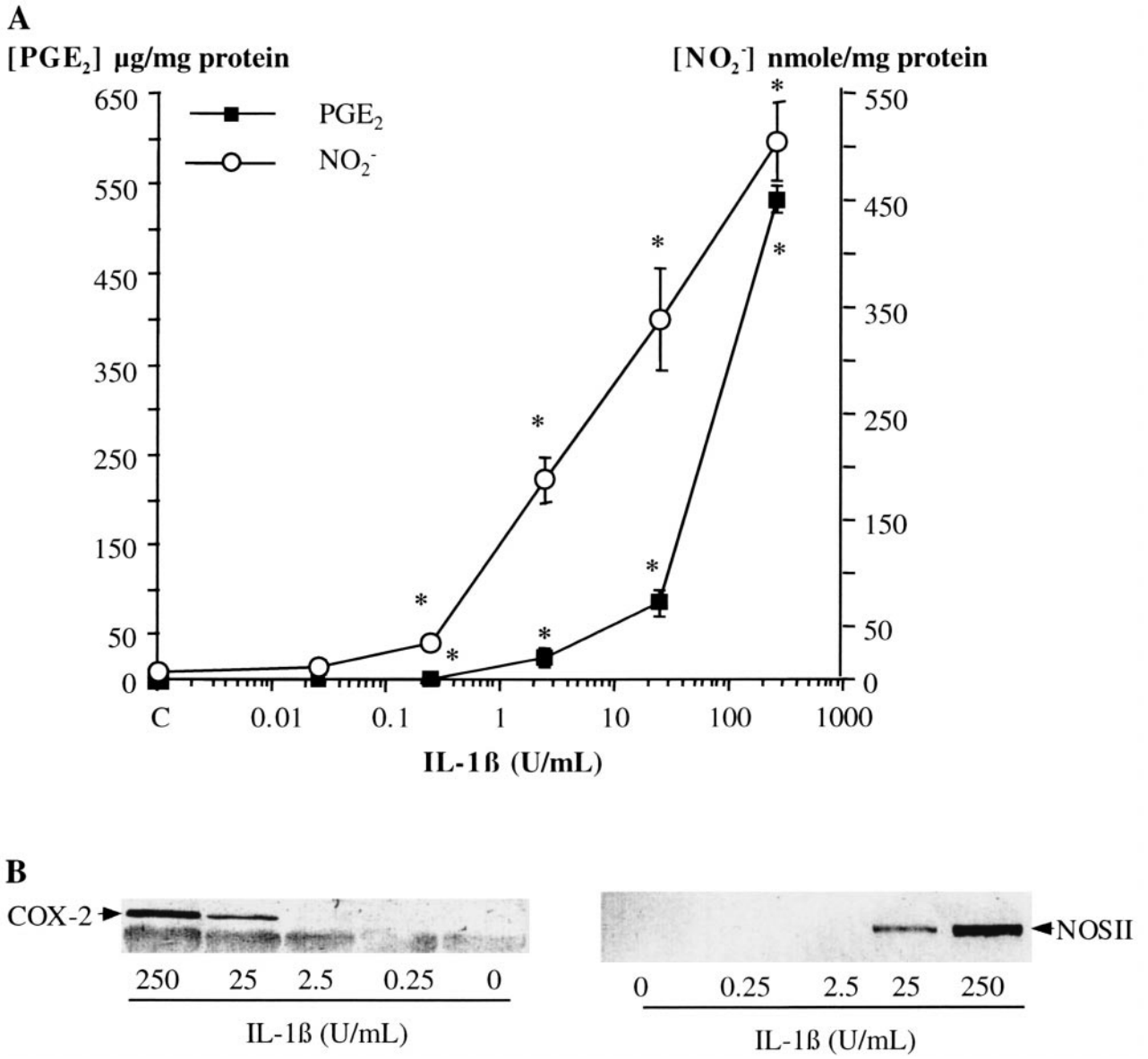

Fig. 1. Dose-response study of IL-1β in rat articular chondrocytes: (A) levels of PGE_2 and NO_2^-, (B) COX-2 and NOS II proteins. Cells were stimulated for 24 h with IL-1β and PGE_2 and nitrite levels were assessed in culture supernatants by EIA and Griess reaction respectively. Values are mean ± SD of 3 to 6 determinations, C = controls, $*P < 0.05$ in comparison to controls. For western blot analysis, each lane was loaded with 50 μg of protein and blotted as described in materials and methods.

ence of 25 U/mL of IL-1β, although high concentrations (0.1 and 1 mM) were required to observe any significant effect (Fig. 3B). In this concentration range, L-NMMA and SMT inhibited the stimulated production of PGE_2 in a dose dependent manner (Fig. 3A). This inhibitory effect was maintained when compounds were added 8 hr after IL-1 (Fig. 4). In both cases, the inhibitory effect of L-NMMA was partly reversed by 5 mM L-Arg, with a greater efficacy on PGE_2 than on NO_2^- levels, whereas D-NMMA was totally ineffective at the highest concentration used (1 mM) (Fig. 3).

In the presence of CuDips, the stimulating effect of IL-1β on PGE_2 production was fully reduced whereas NO_2^- levels remained unchanged. Such inhibitory effect of CuDips on prostaglandin synthesis was maintained when it was added 8 hr after IL-1β. In the same experimental conditions, catalase slightly reduced PGE_2 levels while NO_2^- levels, remained unaffected. However catalase increased both mediators significantly when it was added 8 hr after IL-1β. Co-treatment with CuDips and catalase mimicked the effect of CuDips alone, i.e., a significant fall in PGE_2 level with a marginal variation of NO_2^- production, whatever the time of treatment (Fig. 4).

The NO donors, SNAP at 1 mM, and SIN-1 at 0.1 mM did not increase the basal PGE_2 synthesis whilst producing much higher levels of NO_2^- than IL-1 (Table 3).

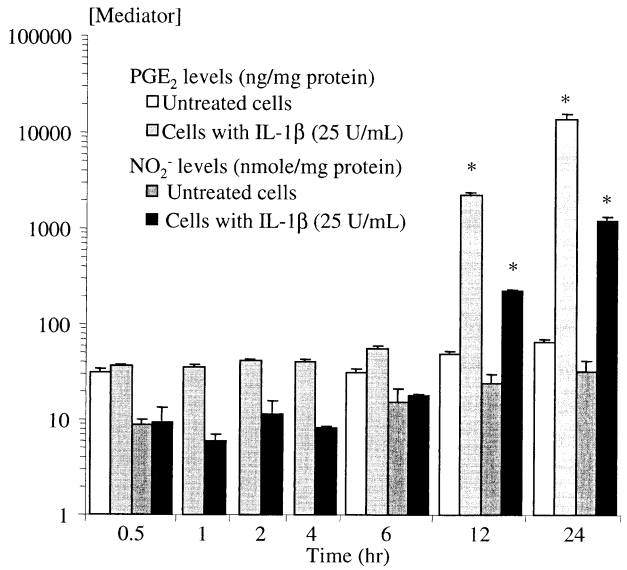

Fig. 2. Kinetics of COX and NOS activities in rat articular chondrocytes stimulated with IL-1β (25 U/mL). At indicated times, culture supernatants were discarded and assayed for PGE_2 and NO_2^- levels by EIA and Griess reaction respectively. Unstimulated cells were used as controls. Values are mean ± SD of 3 to 6 determinations, $*P < 0.05$ in comparison to controls.

3.4. Relevance of COX-2 modulation by NO-derived species in the inhibitory effect of IL-1 on proteoglycan synthesis

Chondrocytes beads maintained in culture for 2 days released spontaneously low amounts of NO_2^- and PGE_2 (data not shown). IL-1β induced a significant increase in both mediators synthesis (Fig. 5A) although the magnitude of its stimulating effect (4 to 5-fold for prostaglandins, 2 to 3-fold for nitrites) was weaker than in monolayers. IL-1 inhibited concomitantly radiolabelled sodium sulphate incorporation and this effect was corrected by L-NMMA and/or CuDips but not by indomethacin and NS-398 (Fig. 5B). L-NMMA reduced NO_2^- and PGE_2 levels to the same extent while CuDips was mostly effective on PGE_2 production. NSAIDs abolished PGE_2 levels without affecting NO production (Fig. 5B). In these experimental conditions, neither L-NMMA, CuDips nor NSAIDs impaired proteoglycan synthesis in unstimulated chondrocytes (data not shown).

4. Discussion

Rat articular chondrocytes produced NO and prostaglandins in a concentration- and time-dependent manner after IL-1β challenge as in other species [2,28–30]. This is consistent with both the restricted basal expression of COX-2 protein in articular tissues and its extensive expression in inflammatory conditions [8,9]. COX-2 and NOS II mRNAs were induced by IL-1β treatment but differed greatly in both their basal expression and time-response to IL-1. In our experimental conditions, *COX2* gene was constitutively expressed in chondrocyte, which is in good accordance with previous studies [2,31] except in humans [3]. In contrast, *NOS II* gene expression failed to be detected in basal conditions as in human chondrocytes [29]. The increase in COX-2 mRNA after IL-1β challenge agreed well with that reported in humans [5], as was also the case for the slow induction of NOS II mRNA expression [29]. Their sustained expression showed that *COX2* and *NOS II* have a long duration which distinguishes them from most other IL-1-induced genes [3,29]. *COX2* gene was up-regulated by the translational inhibitor CHX as reported previously [2], possibly as a result of the stabilization of mRNAs or the modulation of transcription factor activity [32]. When chondrocytes pre-treated with CHX were stimulated with IL-1β, a weak inhibition of COX-2 mRNA expression was observed confirming that the stimulating effect of IL-1β on *COX-2* gene does not require necessarily *de novo* protein synthesis. Post-treatment with ACT D inhibited the induction of COX-2 mRNAs by IL-1β, which is consistent with the 50% decrease reported in rabbit [2]. As COX-2 mRNA steady state level decreased after IL-1 treatment in a similar manner as in basal conditions, it is likely that IL-1 did not act by a stabilisation of the transcripts in rat chondrocyte. *NOS II* expression required *de novo* protein synthesis since

Table 2
Effect of CHX and IL-1β on the regulation of *COX1*, *COX2*, and *NOS II* gene expression in rat chondrocytes

		Interest gene cDNA/G3PDH cDNA (% controls)					
		30 min.	1 hr	2 hr	4 hr	6 hr	24 hr
IL-1β	COX1	135 ± 3	142 ± 11	68 ± 1	100 ± 2	158 ± 32	65 ± 18
(25 U/mL)	COX2	174 ± 16	179 ± 10	259 ± 6	452 ± 40	370 ± 55	360 ± 7
	NOSII	17 ± 3	18 ± 9	104 ± 12	1242 ± 335	1269 ± 384	1150 ± 83
CHX	COX1	67 ± 3	81 ± 13	94 ± 2	55 ± 7	75 ± 14	83 ± 16
(10 μg/mL)	COX2	83 ± 11	74 ± 15	354 ± 26	465 ± 22	392 ± 105	170 ± 28
	NOSII	41 ± 13	56 ± 21	147 ± 50	131 ± 11	382 ± 63	564 ± 12
IL-1β + CHX	COX1	167 ± 27	146 ± 41	47 ± 3	45 ± 8	54 ± 6	47 ± 6
	COX2	167 ± 4	147 ± 30	202 ± 35	375 ± 65	215 ± 20	113 ± 8
	NOSII	64 ± 3	77 ± 8	108 ± 8	130 ± 28	450 ± 40	295 ± 24

Cells were stimulated with either 25 U/mL of IL-1β, 10 μg/mL of CHX (pretreatment 2 hr before medium changing) or both.

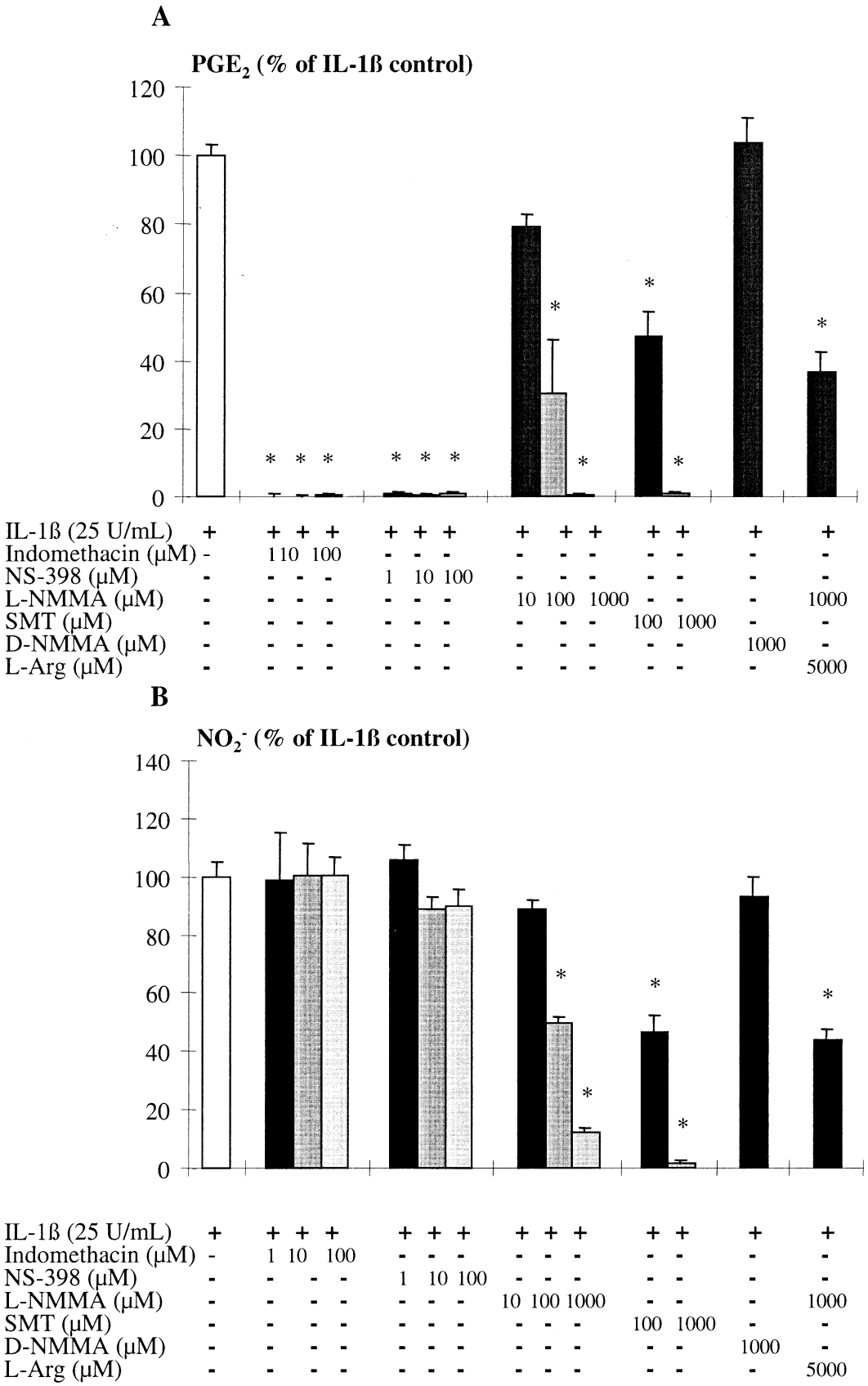

Fig. 3. Effects of NSAIDs and NOS inhibitors on mediators release by rat chondrocytes stimulated for 24 hr with 25 U/mL of IL-1β, (A) PGE_2 levels, (B) NO_2^- levels. Values are mean ± SD of 4 to 6 determinations, *$P < 0.05$ in comparison to IL-1β controls.

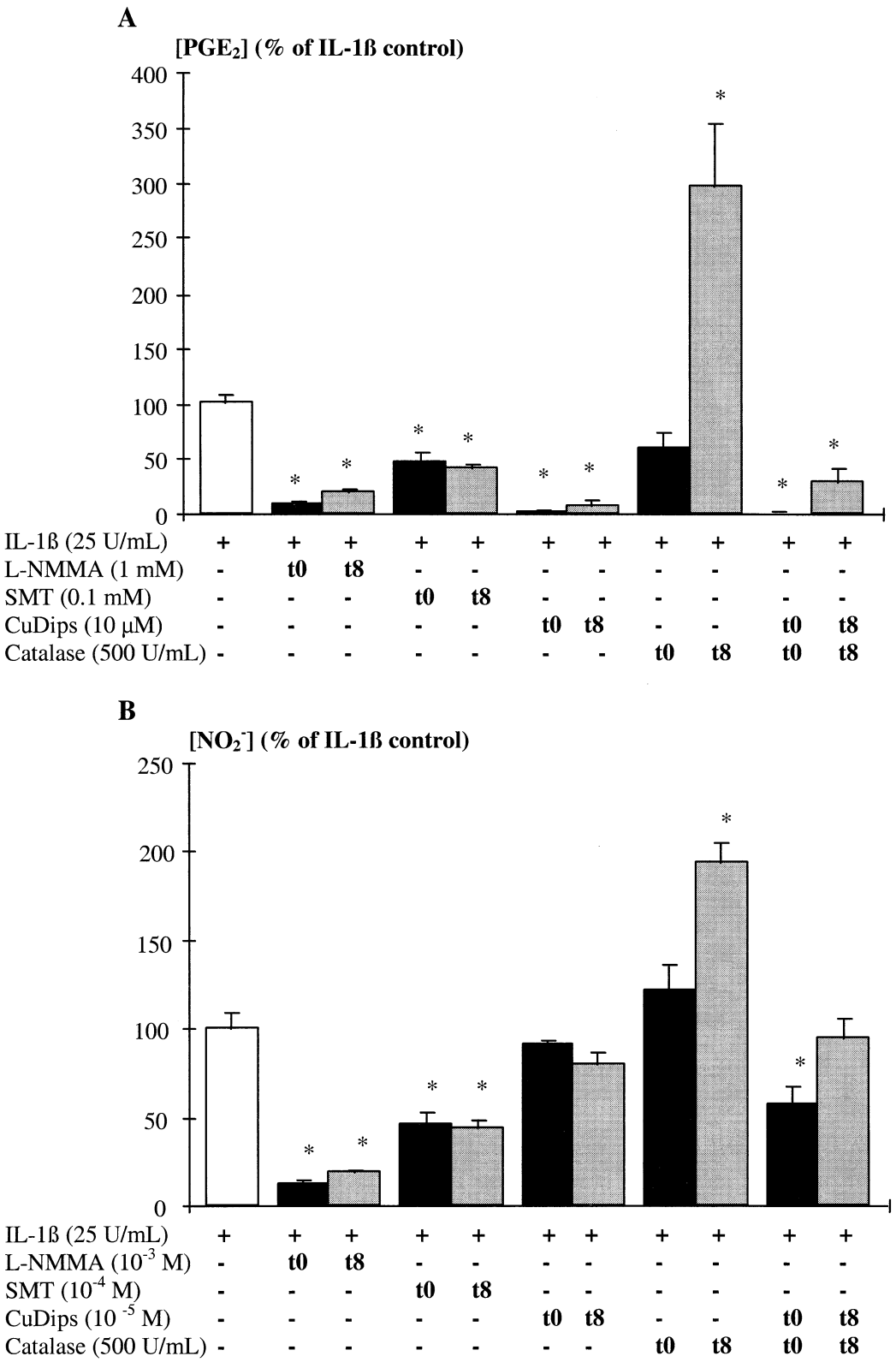

Fig. 4. Effects of NOS inhibitors (L-NMMA, SMT) and radical scavengers (CuDips, catalase) on COX-2 and NOS II activities in rat chondrocytes. (A) PGE_2 levels, (B) NO_2^- levels. Cells were stimulated with IL-1β (25 U/mL) and compounds were added to culture medium concomitantly or at a time (8 hr) allowing a complete expression of both enzymes. Values are mean ± SD of 4 to 8 determinations, $^*P < 0.05$ in comparison to IL-1β controls.

Table 3
Effect of NO donors on basal COX activity in rat chondrocytes

	PGE_2 (ng/mg protein)	NO_2^- (nmole/mg protein)
Controls	16.0 ± 0.4	5.28 ± 0.69
SNAP (1 mM)	21.6 ± 7.2	717.02 ± 46.03*
SIN-1 (0.1 mM)	17.6 ± 4.0	354.69 ± 53.34*

Cells were exposed with SNAP or SIN-1 during 24 hr. Culture supernatants were discarded and asseyed for PGE_2 and NO_2^- levels by EIA and Griess reaction respectively. Values are mean ± SD of 6 to 8 determinations; $*P < 0.05$ in comparison to controls.

CHX reduced the stimulating effect of IL-1 on this gene. In addition, the total suppression of IL-1β-induced *NOS II* gene expression by ACT D suggested that enhancement of transcription may be the major way for the stimulatory effect of IL-1.

The simultaneous increase in PGE_2 and NO_2^- release after IL-1β challenge suggested a cross-talk between NOS and COX pathways. NSAIDs having a different selectivity for COXs isoenzymes *in vitro*, namely indomethacin as a preferential COX-1 inhibitor [1] and NS-398 as a selective COX-2 inhibitor [34], never affected IL-1β-induced NO_2^- release. The concentrations required for inhibiting PGE_2 production were low in comparison to the values of IC_{50} reported for COXs isoenzymes in other systems including human chondrocytes [28], but can be explained by the lack of exogenous arachidonic acid supply [34]. Inhibition of PGE_2 production was likely mediated at the enzyme level [35] since NSAIDs were only marginally reported to affect cellular signaling and, otherwise, at much higher concentrations [36]. Inability of NSAIDs to modulate NO pathway was consistent with data reported in human chondrocytes [37] although NSAIDs were shown, in the millimolar range, to inhibit NO production at a pre-translational level in macrophages [38]. Despite possible expression of EP or IP receptors on chondrocytes [39], our results indicate that secreted prostaglandins failed to regulate NOS activity by a feedback mechanism, as was suggested in other cell types [40].

The non-selective NOS inhibitor L-NMMA and the selective NOS II inhibitor SMT [41] reduced PGE_2 and NO_2^- levels to the same extent, suggesting that NO-derived species have a stimulating effect on COX pathway in the presence of IL-1. A NO-dependent stimulation of PGE_2 synthesis was also reported in human cartilage [30,42] although opposite data were sometimes observed with osteoarthritic samples [9]. As inhibition of COX was maintained when NOS inhibitors were added at a time allowing a complete expression of COX-2 protein (8 hr), this effect is likely due to the modulation of enzyme activity. However, as chondrocytes were also shown to produce high levels of $O_2^{-\cdot}$ [43], H_2O_2 [44] and $OH^{\cdot}$ [45] in response to cytokine stimulation, a possible combination between NO and other radical species has to be considered. This point is crucial because COXs require an hydroperoxide activator to generate the protein tyrosyl radical initiating the oxygenation of arachidonic acid [46]. The low molecular weight SOD mimetic, CuDips, was used to check for the contribution of $O_2^{-\cdot}$ in COX activation, all the more that as it is lipid-soluble, and behaves as an intracellular scavenger [47]. It induced a dramatical reduction of PGE_2 levels demonstrating that COX activation depended strongly on $O_2^{-\cdot}$ tone. At the same time, NO_2^- levels were marginally affected which suggested that COX activation involved NO-derived species, which were poorly reflected by NO_x. As for NOS inhibitors, the decrease in COX activity was maintained when CuDips was added 8 hr after IL-1, showing that the effect was not on the induction of either enzyme. This result agrees with the inability of CuDips to affect transcription when added later than 4 hr after phorbol ester in an *in vivo* model of skin tumor [48]. Interestingly, this SOD mimetic was reported to lack any inhibitory effect on COX activity in purified enzymes [20] and in an *in vivo* model of colonocytes proliferation [49]. In addition, it was shown to prevent peroxynitrite ($ONOO^-$) formation in conditions of vascular oxidant stress [50]. Thus, the common effect of NOS inhibitors and CuDips suggests that the stimulation of COX activity is supported by the formation of $ONOO^-$.

COX-2 was shown to be exquisitely sensitive to the requirement of an hydroperoxide initiator [51] and H_2O_2 was shown to accumulate possibly in the presence of CuDips [47]. Since chondrocytes produced also H_2O_2 in response to cytokines [44], the inactivation of COX-2 have may resulted from a high H_2O_2 levels [52]. This hypothesis agrees well with the ability of catalase to increase COX-2 activity when added at a time (8 hr) allowing a consistent expression of the enzyme. Nonetheless, the inhibitory effect of CuDips on COX-2 was not affected by co-treatment with catalase, demonstrating that it was not supported by an enhanced H_2O_2 production. In order to investigate whether COX-1 activity could be modulated in the same way by NO-derived species, we studied the effect of NO donors on resting chondrocytes. SIN-1 produces spontaneously $O_2^{-\cdot}$ and NO, and SNAP releases NO only but can lead to the formation of peroxynitrite due to the spontaneous generation of radicals by chondrocytes [43,45]. Both compounds failed to increase the basal PGE_2 levels indicating that COX-1 activity was not affected by NO-derived species in our experimental conditions. As peroxynitrite was shown to activate purified COX-1 [53], this may simply reflect the formation of low amounts of $ONOO^-$, all the more that COX-1 requires a 10-fold higher level of hydroperoxide initiator than COX-2 [51]. Alternatively, this result suggests that both isoenzymes could be distinctly affected by NO-derived species in chondrocytes, as was recently reported for NO gas in macrophages [54] and SNAP in synovial cells [55]. However, one cannot rule out that chondrocyte could be more susceptible to endogenously generated NO-derivatives than to exogenously supplied NO-derivatives, as was suggested for glycosaminoglycan synthesis [56]. Taken together, our data demonstrate that COX-2 but not COX-1

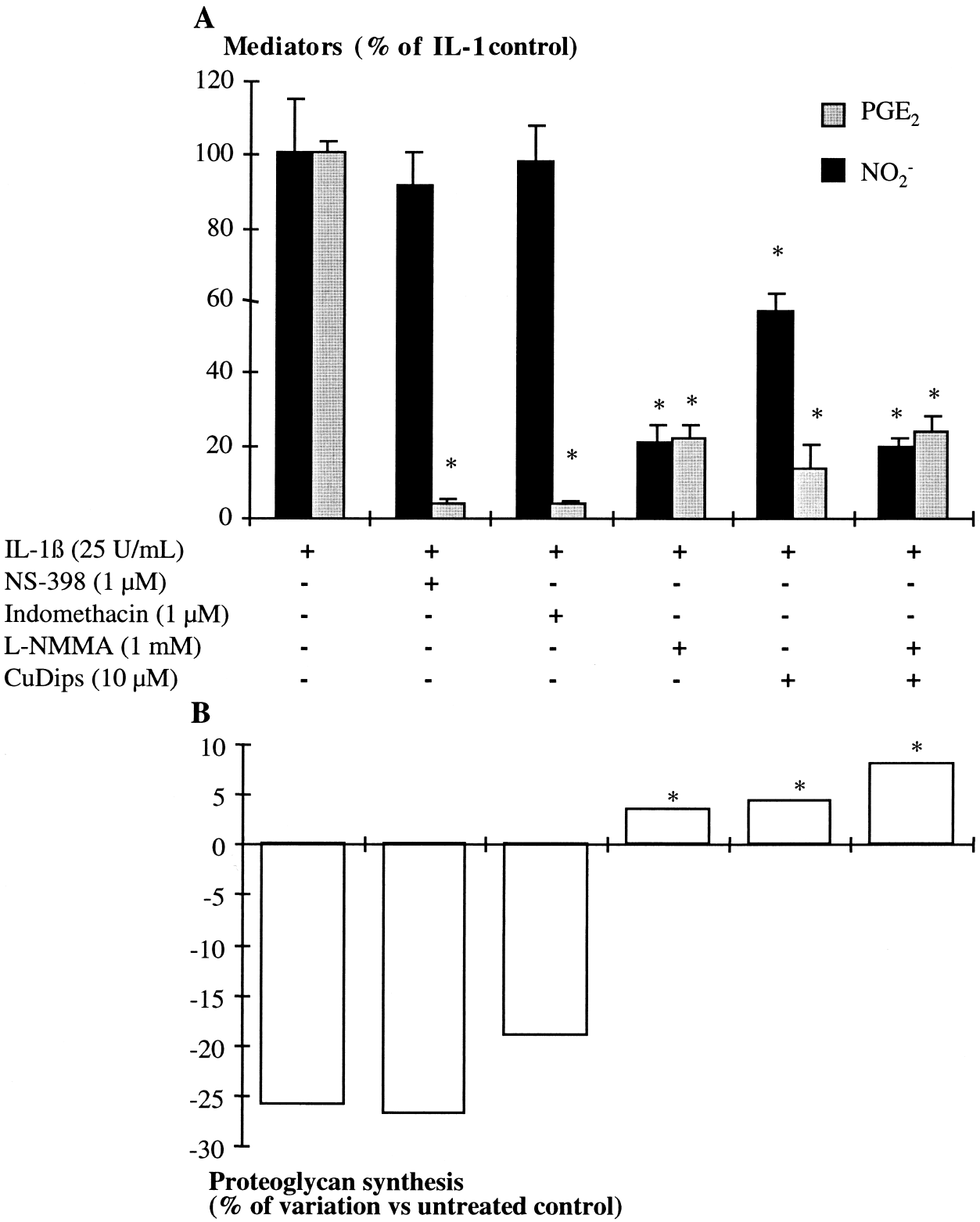

Fig. 5. Contribution of COX-2 and NOS II-derived species to cartilage anabolism: (A) NO_2^- and PGE_2 levels, (B) proteoglycan synthesis. Chondrocytes embedded in alginate were stimulated for 48 hr with IL-1β (25 U/mL) in the presence of NSAIDs, NOS inhibitor (L-NMMA) or SOD mimetic (CuDips) at concentrations inhibiting significantly enzymes activities. Proteoglycan synthesis was assessed by radiolabelled sulphate incorporation as described in materials and methods. Values are mean ± SD of 4 to 8 determinations, $^*P < 0.05$ in comparison to IL-1β controls.

activity is stimulated by NO-derived species in rat chondrocyte, which is consistent with the *in vivo* modulation of eicosanoids production reported in iNOS-deficient mice [57].

Finally, to search for biological consequences of COX-2 modulation by NO-derived species, we investigated the IL-1-induced loss of proteoglycan synthesis in chondrocytes beads. It is now widely accepted that NO contributes to the deleterious effect of IL-1 on cartilage anabolism since NOS inhibitors were able to reverse, at least in part, inhibition of proteoglycan synthesis *in vitro* [56] and *in vivo* [15]. Besides, reactive oxygen species, and especially H_2O_2 generated by the xanthine oxidase and hypoxanthine system, were also shown to suppress proteoglycan synthesis [58]. Based on these data, we suggested that NO-derived species might be effectors of both COX-2 activation and inhibition of proteoglycan synthesis. In chondrocytes stimulated with IL-1, L-NMMA and CuDips inhibited PGE_2 levels while restoring proteoglycan synthesis, raising the possibility that PGE_2 could mediate proteoglycan loss. Indeed, exogenous PGE_2 was shown to inhibit keratan sulphate [59] and aggrecan synthesis [60] in chondrocytes. However, when PGE_2 levels were completely suppressed by NSAIDs, radiolabelled sulphate incorporation did not change significantly in the presence of IL-1. These data demonstrate that endogenous prostaglandins do not play a crucial role in the IL-1 mediated inhibition of glycosaminoglycan synthesis. Another interesting result is that L-NMMA and CuDips restored proteoglycan synthesis to the same extent and that their effects were not additive. As discussed above, this suggests that both compounds may inhibit $ONOO^-$ formation as a common way and that peroxynitrite supports most of the IL-1-mediated reduction of cartilage anabolism. Furthermore, any accumulation of H_2O_2 in the presence of CuDips is unlikely because it would have led to inhibition of proteoglycan synthesis [61]. Recently, nitrotyrosine, a hallmark of peroxynitrite generation, was found in cartilage during experimental osteoarthritis [18], and chondrocyte survival was previously shown to depend on the ability of NO to combine with radical species [62]. Moreover, the dual generation of NO and $O_2^{-\cdot}$ by SIN-1 has been associated with an inhibition of proteoglycan synthesis in unstimulated bovine chondrocytes [63]. Taken together, our data show that NO-derived species induced by IL-1 are involved in the regulation of prostaglandin release and proteoglycan synthesis in chondrocytes but that both effects are not reciprocally causative.

In summary, the present study demonstrates the sequential and distinct activation of *COX-2* and *NOS II* genes by IL-1β and the distinct modulation of COX activity by NO-derived species in rat chondrocytes. Peroxynitrite accounts likely for the stimulation of COX-2 activity and the loss of proteoglycan synthesis induced by IL-1β in chondrocytes, but endogenous prostaglandins synthesis do not participate in cartilage anabolism. These data underline that NO-derived species play a major role in chondrocyte functions, and suggest that their inhibition by scavengers may have therapeutical relevance in joint diseases.

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