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Regulation by reactive oxygen species of interleukin-1β, nitric oxide and prostaglandin E_2 production by human chondrocytes

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Summary

Objectives: To determine the effects of two drugs, N-monomethyl-L-arginine (L-NMMA) and N-acetylcysteine (NAC), on interleukin-1β (IL-1β), nitric oxide (NO) and prostaglandin E_2 (PGE_2) production by human chondrocytes. The effect of aceclofenac (ACECLO), a non-steroidal antiinflammatory drug (NSAID), was also examined.

Methods: Human chondrocytes were enzymatically isolated from osteoarthritic knee cartilage and then maintained in culture in suspension for 48 h in the absence or in the presence of lipopolysaccharide (LPS) (10 μg/ml), L-NMMA (0.5 mM), NAC (1 mM) or ACECLO ($6 \cdot 10^{-6}$ M). IL-1β and PGE_2 productions were quantified by specific immunoassays. Nitrite was measured in the culture supernatants by a spectrophotometric method based upon the Griess reaction. Cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS) and IL-1β gene expressions were quantified by transcription of mRNA followed by real time and quantitative polymerase chain reaction. COX-2 protein expression was analysed by Western blot.

Results: LPS markedly increased the expression of IL-1β, iNOS and COX-2 genes. In parallel, NO_2 and PGE_2 amounts found in the culture supernatants were significantly enhanced whereas IL-1β was immunologically undetectable. The addition of L-NMMA (0.5 mM) fully blocked LPS-induced NO production but greatly increased PGE_2 production, suggesting a negative effect of NO on PGE_2 synthesis. Inversely, NO production was stimulated by NAC while PGE_2 production was not affected. Interestingly, NAC increased the IL-1β and iNOS mRNA levels but did not significantly modify COX-2 mRNA expression. L-NMMA did not significantly affect the expression of IL-1β, iNOS and COX-2. The amount of COX-2 protein did not change in the presence of the antioxidants. Finally, ACECLO fully blocked the production of PGE_2 by chondrocytes without affecting the levels of COX-2 mRNA.

Conclusions: The stimulation of IL-1β, NO and PGE_2 production by LPS is differentially controlled by reactive oxygen species (ROS). In fact, L-NMMA and NAC have different mechanisms of action on the regulation of NO and PGE_2 productions. L-NMMA fully inhibits NO but increases PGE_2 production whereas NAC up-regulates NO but does not modify PGE_2 synthesis. The stimulating effect of L-NMMA on PGE_2 production is not controlled at the transcriptional level. These findings suggest that antioxidant therapy could have different effects according to the oxygen radical species targeted.

Key words: Reactive oxygen species, Chondrocyte, Nitric oxide, Cytokines, Arthritis.

Introduction

NO is a gaseous free radical, which is transformed to nitrite and nitrate in the presence of oxygen. NO is synthesized through L-arginine oxidation by a family of NO synthases. It rapidly reacts with superoxide anions ($O_2^{\cdot-}$) to form peroxynitrite ($OONO^-$) which is a strong oxidant. It is well accepted that chondrocytes possess a NADPH oxidase and produce both ROS and NO[1,2]. *In vitro*, ROS and NO productions are strongly stimulated by IL-1β, which is a key cytokine in the pathogenesis of arthritis[3]. Nitric oxide is presented as a catabolic agent since it was demonstrated that intraarticular injection of L-NIL (n-iminoethyl-L-lysine), a selective inhibitor of inducible nitric oxide synthase, reduced the progression of cartilage erosion in an experimental osteoarthritic dog model[4]. *In vitro*, the inhibition of endogenous NO production by L-NMMA, another well established NO synthase inhibitor, led to the suppression of IL-1β stimulating effects on metalloproteases[5] and partially reversed the IL-1β inhibitory effect on glycosaminoglycan synthesis by chondrocytes[6]. Furthermore, NO inhibits IL-1 receptor antagonist (IL-1ra) production by chondrocytes, a mechanism possibly responsible for the enhancement of IL-1 biological activity[7].

Although there is a general agreement that NO is involved in cartilage destruction, the literature is unclear about the role played by NO in the initiation and progression of synovial membrane inflammation. NO was originally identified as the endothelium-derived relaxing factor with potential proinflammatory effects, including increase of vascular permeability, enhancement of PGE_2 synthesis[8–10] and the induction of catabolic cytokines like IL-1β and

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TNFα. Further evidence that NO may be antiinflammatory under certain circumstances is provided by its ability to down-regulate PGE_2[11–14], IL-6 and IL-8 production by chondrocytes and liver kupffer cells[13,15] and to suppress the production of $O_2^{\cdot-}$ by neutrophils through a direct action upon NADPH-oxidase[16]. These conflicting results obtained with NO synthase inhibitor could be explained by the fact that intracellular signalling pathways are very sensitive to intracellular fluctuation of ROS levels. This hypothesis gains strong support from the finding that the two well studied nuclear transcription factors, NF-kB and AP-1, are activated when intracellular ROS are increased or decreased, respectively[17]. These factors are involved in rapid induction of gene transcription in response to a variety of pathogenic conditions, specifically chronic inflammation. Another explanation could be that NO reactivity could be strongly different according to its ability to react with other oxygen species to form new oxidative molecules. In fact, NO interacts easily with $O_2^{\cdot-}$ to form not only the most potent peroxynitrite but also derivative active free radicals such as peroxynitrous acid, hydroxyl radical and nitrogen dioxide[18].

This complex interdependent regulation of NO, IL-1β and PGE_2 creates a dilemma for pharmacological intervention of NO. In pathological context, such as arthritis where both inducible NOS and COX-2 are activated, therapeutical strategies focused on regulating COX activity by NO may be of some benefit.

In view of these observations, this study was designed to investigate the effects of antioxidant molecules on IL-1β and PGE_2 productions by human chondrocytes and then to identify the regulatory role played by NO or derived reactive species.

Materials and methods

SHORT TERM SUSPENSION CULTURE OF HUMAN CHONDROCYTES

Cartilage specimens were obtained from the knees of eight donors shortly after death (four males and four females) with the mean age of 49 (47–52) years old, being excised from the superficial and medium layers and avoiding the calcified layer. Upon dissection, femoral and patellar articular surfaces were evaluated for the severity of the macroscopic cartilage lesions using a personal scale. Four different grades were considered: 0, normal white cartilage on all areas examined; I, presence of yellow-gray area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces; III, ulcers penetrating to subchondral bone on one or more articular surface. Four out of the eight cartilage samples collected showed lesion of grade III and four cartilage samples had lesion of grade I. Each culture was run with chondrocytes from a single patient. The cartilage slices were cut into small fragments and then subjected to sequential enzymatic digestions with hyaluronidase, pronase and collagenase as previously described[13]. The cells were then filtered through a nylon mesh (70 μm), washed three times, counted (1.10^6 cells/ml) and resuspended in 1 ml of adequate culture medium: DMEM without red phenol (Biowhittaker, Brussels, Belgium) supplemented with 10 mM HEPES, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 1% of ITS^+ (ICN, Doornveld, Belgium). ITS^+ is a culture supplement containing in 1 ml: 0.625 mg of insulin, 0.625 mg of transferrin, 0.625 μg of selenious acid, 125 mg of bovine serum albumin (BSA) and 0.535 mg of linoleic acid. The cells recovered after collagenase digestion of the tissue were 95% viable (trypan blue test). Cells were then kept in culture medium for 48 h in order to eliminate *in vivo* contamination with drugs that the donors may have taken before death.

After this washout period, cells ($1 \cdot 10^6$ cells/ml in culture medium) were seeded in 15 ml polypropylene Falcon tubes and maintained under agitation on a gyratory shaker (100 rpm) in a 95% air/5% CO_2 environment. Chondrocytes were cultured for 48 h in the absence or in the presence of LPS (10 μg/ml, serotype 026:B6; Sigma-Aldrich, Bornem, Belgium) and with or without L-NMMA (0,5 mM; Calbiochem, San Diego, U.S.A.) NAC (1 mM); Sigma-Aldrich, Bornem, Belgium) or ACECLO ($6 \cdot 10^{-6}$ M; Prodesfarma, Spain). ACECLO was first dissolved in N,N-dimethylformamide (DMF, Sigma-Aldrich, Bornem, Belgium), and then diluted in culture medium to achieve the required concentration. The final concentration of DMF was 0.1%. A similar amount of the vehicle was added to the controls. Cells and supernatant were then separated by centrifugation (1000 rpm; 5 min). Other chemicals were of the purest commercial grade available.

NITRIC OXIDE ASSAY

NO production was determined by quantifying its derived product nitrite in conditioned medium using a spectrophotometric method based on the Griess reaction[19]. Briefly, 150 μl of conditioned culture medium or sodium nitrite ($NaNO_2$) standard dilutions were mixed with 100 μl of Griess reagent (0.5% sulfanilanide, 0.05% naphtyl ethylenediamine dihydrochloride, 2.5% H_3PO_4) and incubated for 5 min at 37°C. The limit of detection was 2 μM of nitrite. Before adding the Griess reaction, nitrate was converted to nitrite by treating sample with nitrate reductase (0.2 U/mL, Roche, Brussels, Belgium)[20]. The absorption was measured at 540 nm.

PGE2 RADIOIMMUNOASSAY

PGE_2 level was determined in conditioned culture medium according to a previously described radioimmunoassay (RIA)[21] using a polyclonal antiserum, obtained from rabbit. The ^{3}H-labeled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule PGE_2 from Sigma-Aldrich (Bornem, Belgium). PGE_2 was measured in triplicate in a 100-μl aliquot of each sample. After 48 h of incubation at 4°C, free antigen was separated from antibodies-antigen complex by charcoal precipitation and centrifugation (20 min at 1000 ***g***, 4°C). The supernatant was counted by liquid scintillation. Intra- and interassay coefficients of variation were 6 and 10%, respectively. The recovery of known amounts of PGE_2 added to the culture medium was satisfactory (90 to 97% for the amounts of PGE_2 ranging from 2500 to 312 pg/ml). The limit of detection of the RIA was 20 pg/ml.

IL-1β ASSAYS

IL-1β production by chondrocytes was assayed in the culture media by two different enzyme amplified sensitivity immunoassays (EASIA) designed by RD System (Abingdon, U.K.) and by Genzyme (Cambridge, U.S.A.). The assays were directly performed in the culture media

without previous extraction. The limits of detection of the two assays were 1 and 0.1 pg/ml, respectively.

DNA ASSAY

Chondrocyte DNA content was directly correlated to the cell number of each culture. DNA content was measured in the cell pellet extracts using a fluorimetric method. This measurement ensures elimination of result variations due to the different number of chondrocytes[22].

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RNA was isolated by a single step guanidinium thiocyanate-phenol-chloroform method. Cells ($3 \cdot 10^6$) were lysed using TRI REAGENT (Sigma-Aldrich, Bornem, Belgium) and the samples were processed following the manufacturer's protocol. RNA (2 μg) was reverse transcribed in a volume of 30 μl containing 1× RT buffer, 10 mM DTT, 1 mM dNTPs, 500 ng oligo$(dT)_{15}$ primer, 140 U M-MLV reverse transcriptase and 40 U RNaseOUT (Life Technologies, Merelbeke, Belgium) for 60 min at 37°C. The reaction was halted by an incubation at 95°C for 5 min and by adding 100 μl of H_2O.

The polymerase chain reaction was carried out in an automatic DNA thermal cycler (Hybaid, Doornveld, Belgium). Primer sequences used to amplify the desired cDNA are as follows: hβ-actin forward and reverse primers: 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAAT GTCACGCACGATTTC-3′; hGAPDH forward and reverse primers: 5′-TTGGTATCGTGGAAGGACTCA-3′ and 5′-TGTCATCATATTTGGCAGGTTT-3′; hiNOS forward and reverse primers: 5′-CCATGGAACATCCC AAATAC-3′ and 5′-TCTGCATGTACTTCATGAAGG-3′; hCOX-2 forward and reverse primers: 5′-TTCAAATGAGATTGTGGGA AAA-3′ and 5′-AGATCATCTCTGCCTGAGTATCTT-3′; h IL-1β forward and reverse primers: 5′-TCTCCGACC ACCACTACAGCA A-3′ and 5′-GGGGAACTGGGCAGA CTCA A-3′.

The reaction mixture contained 2 μl cDNA (equivalent to 30 ng of RNA), 0.5 μM sense and antisense primer, 250 μM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$ and 1U Taq DNA polymerase (Life Technologies, Merelbeke, Belgium) in a final volume of 40 μl. The cycle program was set to denature at 95°C for 45 s, to anneal at 55°C for 45 s, and to extend at 72°C for 90 s. The number of cycles was selected in the exponential phase of the amplification curve (20 cycles for β-actin, 26 for iNOS, COX-2 and IL-1β).

Electrophoresis of the PCR products was performed on 1.5% agarose gels containing 0.1 μg/ml ethidium bromide.

QUANTITATIVE REAL-TIME PCR

PCR reaction was performed by using the LightCycler-FastStart DNA master Sybr Green I (Roche Diagnostics, Brussels, Belgium). This reaction mixture is provided as a 10-fold stock solution containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix, 10 mM $MgCl_2$ and SYBR Green I dye. For each LightCycler PCR, a mastermix of the following reaction components was prepared in a final volume of 18 μl: 2 μl LightCycler-FastStart DNA master Sybr Green I, 1.6 μl of 25 mM $MgCl_2$, 0.5 μM sense and antisense primer. The mastermix was loaded into glass capillary (Roche Diagnostics, Brussels, Belgium) and 2 μl of PCR template was added. The template source was either 3 ng first-strand cDNA or purified DNA standard.

Amplification was performed with a spectrofluorometric thermal cycler (LightCycler, Roche Diagnostics, Brussels, Belgium). After an initial denaturation step at 95°C for 10 min, amplification was performed using 40 cycles of denaturation (95°C for 15 s), annealing (temperature gradient: 68°C to 58°C with an increment of 0.5°C per cycle during 20 cycles, continued by 20 cycles at 58°C, for 5 s) and extension (72°C for 15 s). For each run, a standard curve was generated from purified DNA ranging from 10^6 to 10 copies. To standardize mRNA levels, we amplified glyceraldehyde-3-phosphate deshydrogenase (GAPDH), a house-keeping gene as internal control. Normalized gene expression was calculated as the ratio between sample and GAPDH cDNA copy number.

After amplification, a final melting curve was recorded by cooling the PCR mixture to 65°C for 30 s and then slowly heating it to 95°C at 0.1°C/s. Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of the dsDNA. Specificity of the expected IL-1β, iNOS and COX-2 LightCycler products were demonstrated by melting curves analysis.

Amplification products performed in the LightCycler were checked by electrophoresis on 1.5% ethidium bromide stained agarose gel. The estimated size of the amplified fragments matched the calculated size.

WESTERN-BLOT ANALYSIS

After incubation with the different drugs, chondrocytes ($5 \cdot 10^6$ cells) were washed three times with PBS and lysed by boiling for 5 min in 150 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 0.1 mg/ml PMSF). Equal amounts of protein (20 μg) estimated by bicinchonimic acid reagent (Pierce, Rockford, U.S.A.) were loaded onto 10% SDS-polyacrylamide gel and were transferred to nitrocellulose membrane (Bio-Rad, Nazareth, Belgium). Sheep COX-2 purified enzyme (Cayman Chemical, Ann Arbor, U.S.A.) was also loaded on the gel as positive control. The membrane was satured with 5% fat-free dry milk in phosphate-buffered saline (80 mM Na_2HPO_4, 25 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) with 1% (v/v) Tween 20 (PBST) overnight at 4°C. The membrane was incubated with purified polyclonal rabbit IgG antibody against a peptide corresponding to amino acids 567–599 of human COX-2 (Cayman Chemical, Ann Arbor, U.S.A.) at 1:1000 dilution in PBST for 1 h at room temperature. The blot was washed in PBST three times (10 min each). The blot was further incubated for 1 h at room temperature with goat anti-rabbit IgG antibody coupled to horseradish peroxidase (Biosource, Nivelles, Belgium) at 1:20,000 dilution in PBST. After three washing (10 min) in PBST, the blot was incubated with chemiluminescence substrates (ECL Plus, Amersham, Rosendaal, The Netherlands) according to the manufacturer's instructions. The blot was exposed to a radiographic film for 15 s.

CALCULATION AND STATISTICAL ANALYSIS

The results were expressed as amounts of NO_2 plus NO_3 and PGE_2 into supernatants per μg of DNA. The mean±standard deviation (S.D.) of each variable was calculated. A multivariate repeated measures ANOVA model with calculation of contrast was performed on all of the experiments (MANOVA; SAS system; general linear models).

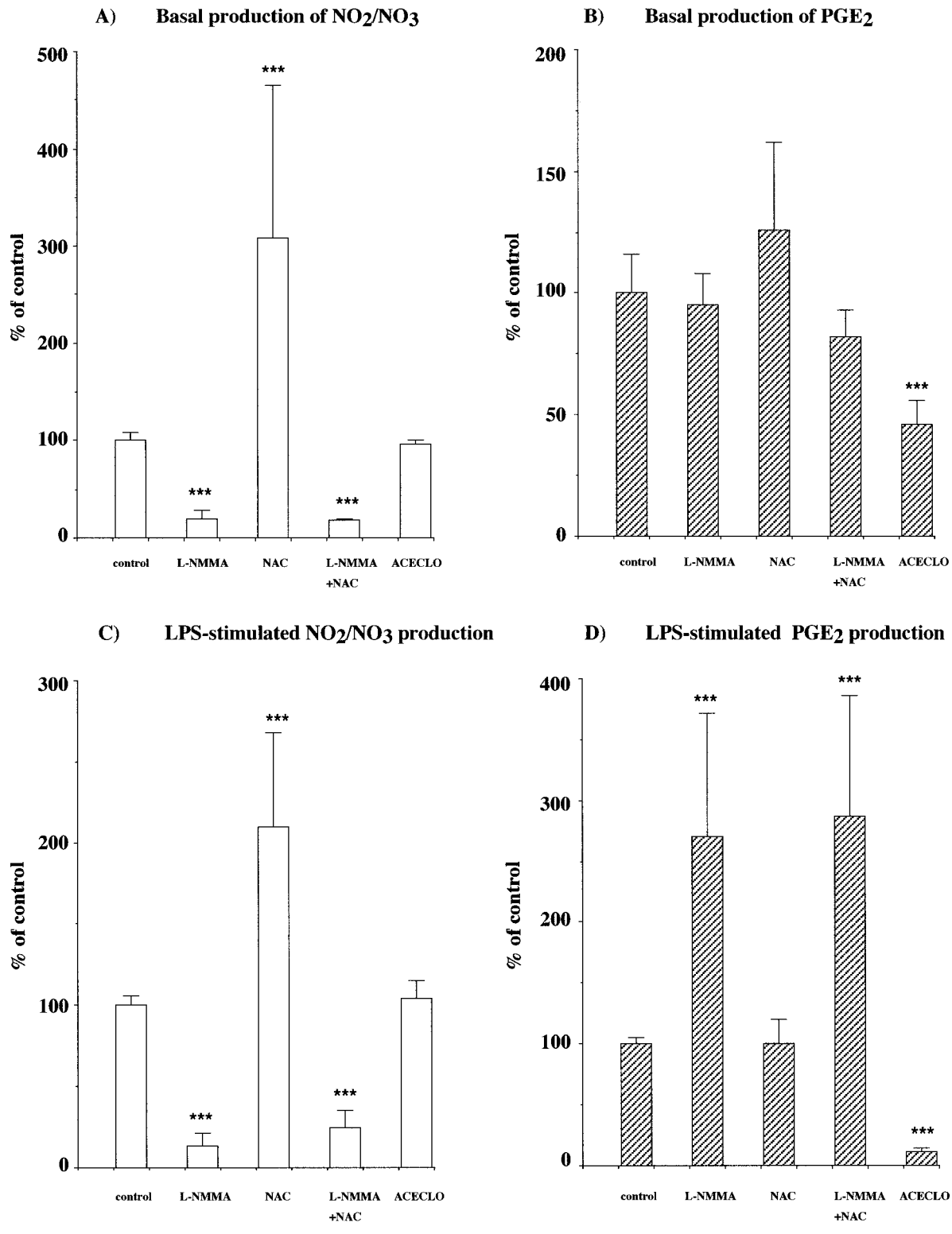

Fig. 1. Effects of L-NMMA, NAC and ACECLO on the productions of NO_2/NO_3 and PGE_2 by human chondrocytes. Chondrocytes were cultured for 48 h in the absence (A), (B) or the presence (C), (D) of LPS (10 μg/ml) and with or without with L-NMMA (0.5 mM), NAC (1 mM), L-NMMA (0.5 mM)+NAC (1 mM) or ACECLO ($6 \cdot 10^{-6}$ M). NO_2/NO_3 [white columns, (A) and (C)] and PGE_2 [hatched columns, (B) and (D)] concentrations were measured in conditioned culture supernatants and are normalized to the production obtained in the absence of the drug. Each condition was tested in triplicate. Results are the mean±S.D. of seven experiments performed with cartilage specimens of different donors. Comparison of mean values was performed by ANOVA analysis. Drug treated groups are significantly different from the untreated groups: ***P<0.001.

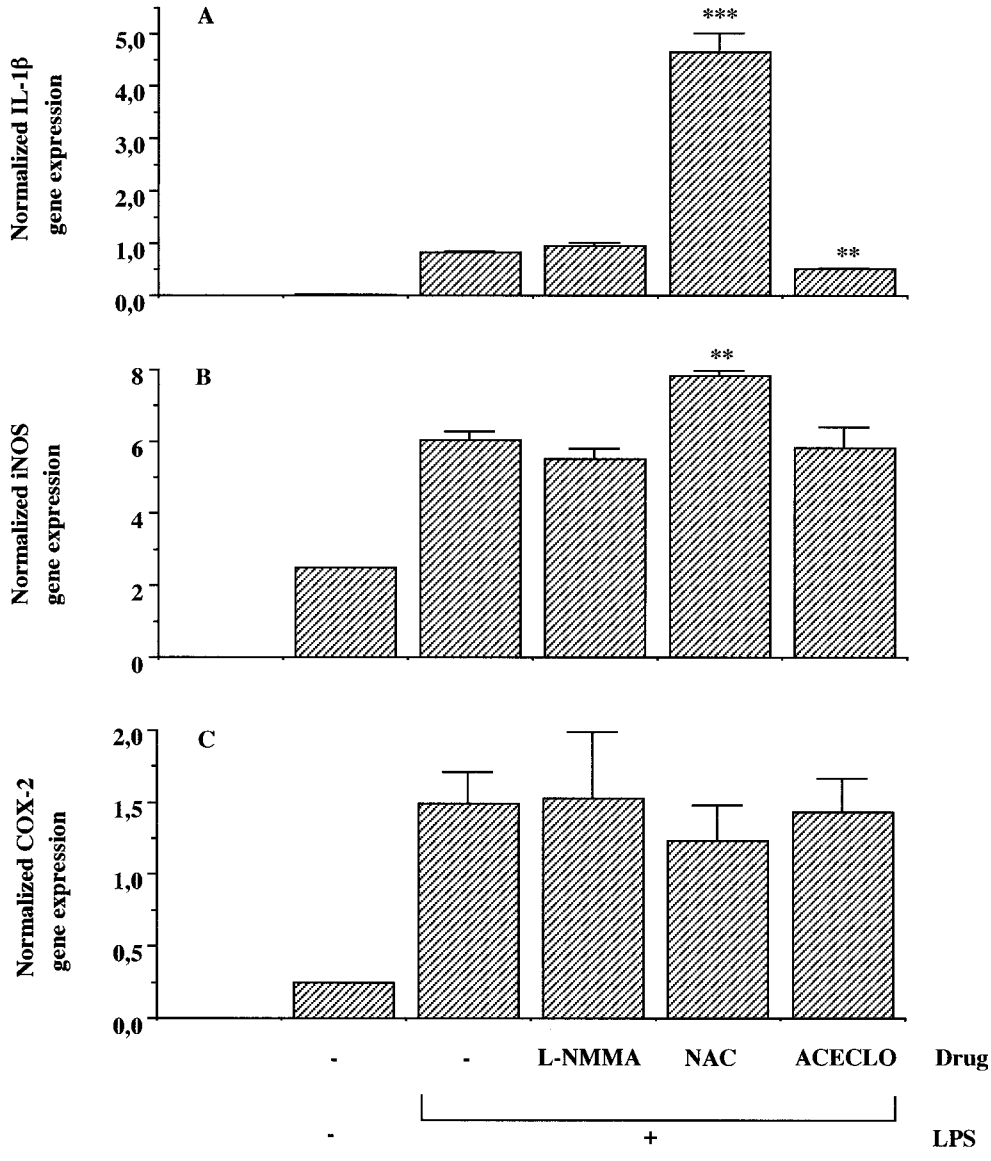

Fig. 2. Effect of L-NMMA, NAC and ACECLO on IL-1β, iNOS and COX-2 mRNA levels. Human chondrocytes were cultured for 48 h in the absence or the presence of LPS (10 μg/ml). Co-incubation with L-NMMA (0.5 mM), NAC (1 mM) or ACECLO ($6 \cdot 10^{-6}$ M) were realized. Total RNA were isolated and mRNA of IL-1β (A), iNOS (B), COX-2 (C) quantified by real-time RT PCR as described in Materials and Methods. The mRNA copy numbers were normalized against the corresponding copy number of GAPDH mRNA. Data represent mean±S.D. of triplicate. Comparison of mean values was performed by ANOVA analysis (drug treated groups are significantly different from the untreated groups: ***P<0.001, **P<0.01).

Results

EFFECTS OF L-NMMA, NAC AND ACECLO ON NO AND PGE2 PRODUCTIONS BY HUMAN CHONDROCYTES

Chondrocytes were isolated from seven different donors. As previously described[13], NO and PGE_2 synthesis were significantly stimulated in the presence of LPS. In both basal and LPS-stimulated conditions the NO and PGE_2 productions varied markedly according to the donor considered. After 48 h of incubation, the mean basal production of NO_2/NO_3 and PGE_2, calculated on seven different chondrocyte cultures, were 2.39 nmoles/μg DNA/48 h and 10.41 pg/μg DNA/48 h, respectively. LPS increased NO and PGE_2 productions by mean 2.8 and 7.2 times, respectively. Whichever donor was considered, IL-1β was undetable.

As expected, L-NMMA reduced by 81 and 87% the basal and LPS-stimulated NO production, respectively [Fig. 1(A),(C)] (P<0·001). As previously described[13], the addition of L-NMMA resulted in a significant enhancement of LPS-stimulated PGE_2 production [Fig. 1(D)] (P<0.001), suggesting a negative effect of NO on PGE_2 synthesis.

Inversely, NAC increased both the basal and LPS-stimulated NO production [Fig. 1(A),(C)] without significant effect on PGE_2 synthesis (Fig. 1(B),(D)]. When NAC and L-NMMA were added simultaneously, NO and PGE_2 productions varied in the same order of magnitude that when L-NMMA was added alone (Fig. 1). ACECLO, a non-steroidal antiinflammatory drug, strongly inhibited

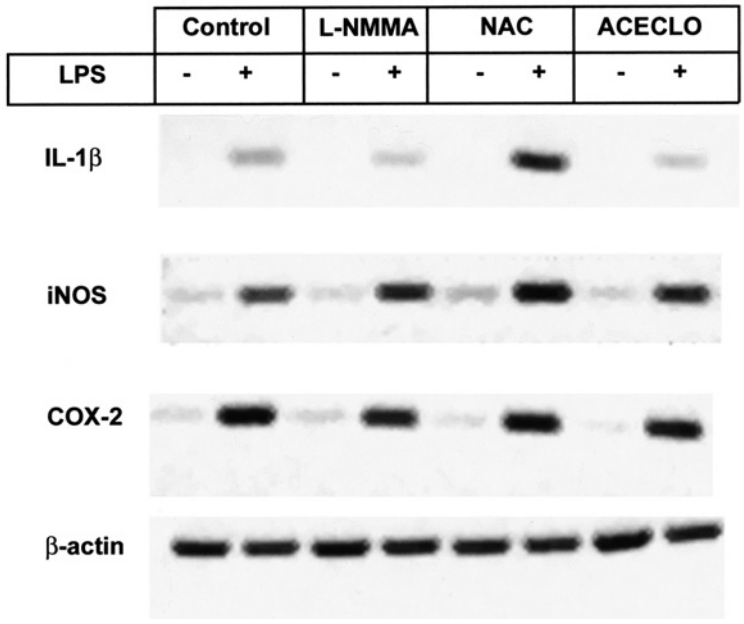

Fig. 3. Effects of L-NMMA (0.5 mM), NAC (1 mM) and ACECLO ($6 \cdot 10^{-6}$ M) on gene expressions. RT-PCR products of IL-1β, iNOS, COX-2 and β-actin were fractionated by electrophoresis through a 1.5% agarose gel and visualized by ethidium bromide staining.

PGE_2 production by both untreated and LPS-treated chondrocytes but did not significantly modify NO production (Fig. 1).

EFFECTS OF L-NMMA, NAC AND ACECLO ON IL-1β, INOS AND COX-2 mRNA EXPRESSION

Real-time RT PCR was performed to quantify the drug effects on gene expression (Fig. 2). Under basal condition, both iNOS and COX-2 mRNA were expressed at low levels while IL-1β mRNA was undetectable. As expected, LPS (10 μg/ml) induced a marked increase of IL-1β, iNOS and COX-2 mRNA levels. After 48 h of incubation, LPS-stimulated chondrocytes expressed until 19 863±642, 112 600±2326 and 11 283±1138 cDNA copies number per 3 ng of total RNA of IL-1 β, iNOS and COX-2 respectively.

L-NMMA (0.5 mM) did not significantly modify LPS-stimulated gene expressions (Fig. 2). NAC (1 mM) enhanced IL-1β ($P<0.001$) and iNOS mRNA levels ($P<0.01$) [Fig. 2(A),(B)] without affecting the COX-2 gene expression [Fig. 2(C)]. Finally, ACECLO ($6 \cdot 10^{-6}$ M) had a significant inhibitory effect on IL-1β gene expression ($P<0.01$) [Fig. 2(A)] but did not affect COX-2 and iNOS mRNA levels. These results were confirmed by classical RT PCR followed by agarose electrophoresis (Fig. 3).

EFFECTS OF L-NMMA AND NAC ON COX-2 PROTEIN

To determine the mechanism of action of L-NMMA on PGE_2 production, we studied the COX-2 protein expression by Western blot analysis (Fig. 4). In the basal condition, COX-2 protein level was very low and was not modified by L-NMMA and NAC treatments. An LPS-inducible expression was observed. L-NMMA and NAC added separately or in combination had no significant effect on COX-2 protein level.

Discussion

We have demonstrated that the inhibition of NO_2 production by L-NMMA was accompanied by an increase of PGE_2 production suggesting a negative feedback of NO on PGE_2 synthesis. Our results are in accordance with previous studies showing by the inhibition of NO synthase or the incubation of cells with NO donors, that NO down-regulated PGE_2 production in cell line murine macrophages[23], in rat peritoneal macrophages[11], in human and bovine chondrocytes[13,14] and in human osteoarthritic cartilage explants[12,14]. In contrast, others have reported that NO enhanced COX-2 activity and PGE_2 production in different cell types[8–10]. Interestingly, Stadler[3] demonstrated a dual role of L-NMMA on PGE_2 production in rabbit chondrocytes culture. When low or high levels of NO were produced by cells activated by, respectively, IL-1β alone or by a combination of IL-1β plus LPS, an inhibition or an enhancement of the PGE_2 production was obtained. Stadler's study provides a possible explanation for the discrepancy reported in the literature about the relation between NO and PGE_2 synthesis, suggesting a biphasic effect of NO according to its production level.

Furthermore, we have shown that L-NMMA did not modify both the COX-2 mRNA level and protein synthesis. These observations suggested that the overproduction of PGE_2 resulting from the L-NMMA treatment was directly related to the control of the enzymatic activity of cyclo-oxygenase. This control could be performed by nitrogen-derived oxidant molecules that could regulate by tyrosine nitration and oxidation the cyclo-oxygenase activity. This mechanism was reported for other enzymes. For example, manganese superoxide dismutase was inactivated by $ONOO^-$ through a mechanism involving not only nitration of critical tyrosine residues but also tyrosine oxidation and subsequent formation of dityrosine[24]. Roy *et al*.[25] demonstrated that NO reacts rapidly with ribonucleotide

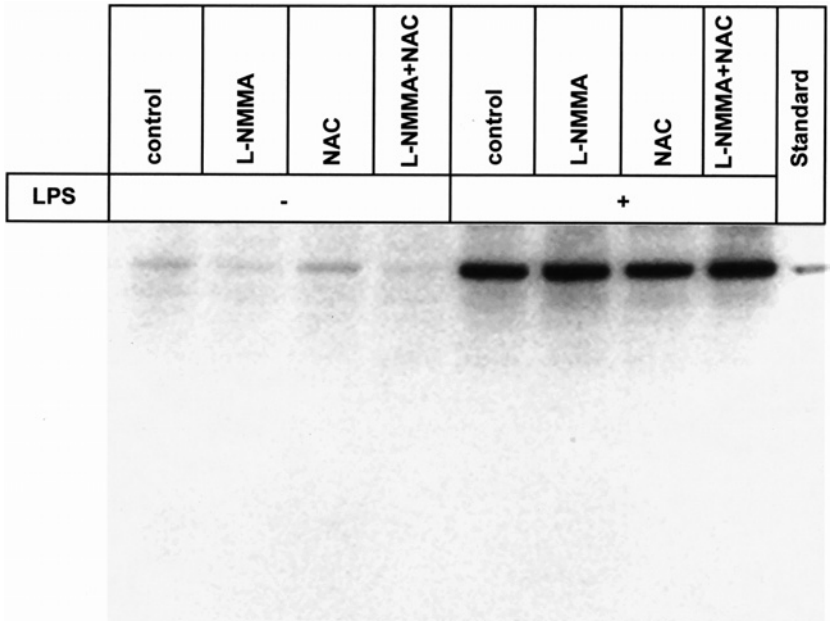

Fig. 4. Western blot analysis of COX-2 in human chondrocyte extracts. Cells were cultured for 48 h in the absence or the presence of LPS (10 μg/ml). L-NMMA (0.5 mM) or NAC (1 mM) were added at the beginning of culture. The cells were lysed and protein extracts were analysed by Western blot with a specific antiserum for COX-2. Ovine COX-2 (400 ng) was used as standard.

reductase, silencing its electronic paramagnetic resonance tyrosyl radical signal and inhibiting its enzymatic activity. COX-2 also produces a tyrosyl radical critical to its catalytic activity[26]. In previous studies, we and others have shown that phorbol myristate acetate, LPS, anoxia–reoxygenation cycles and several cytokines (IL-1β, TNFα, IFNγ), induced the production of ROS by chondrocytes including $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$[1,2]. Furthermore, using RT-PCR, we have observed that in our experimental conditions chondrocytes expressed the p22 sub-unit which is a regulatory element of the NADPH oxidase (data not shown). These data suggest that ROS, produced intracellularly by LPS-stimulated chondrocytes, could react with NO to form $ONOO^{-1}$, which is presented as a potential enzyme inhibitor. Finally, we can also suggest that NO or a nitric-oxide derived oxidant reacts with the phospholipase A2 and inhibits the release of arachidonic acid, the subtrate of cyclo-oxygenases. Nevertheless, Kosonen and colleagues[27] did not confirm this latter hypothesis, showing that the addition of exogenous arachidonic acid to endothelial cell cultures did not alter the inhibitory effect of NO donors on prostacyclin production, suggesting that phospholipases are not the target of NO action. On the other hand, Martinez *et al.*[28] reported an increase of PLA2 activity in the membrane fraction of murine peritoneal macrophages submitted to SIN-1, a $NO/O_2^{\cdot-}$ donor. This stimulating effect was markedly inhibited in the presence of SOD, while L-NMMA had no effect.

In our study, we have also demonstrated that NAC increased NO synthesis and iNOS gene expression but was without effect on PGE_2 production and COX-2 mRNA levels. These findings corroborate the results of Jiang and Brecher[29] demonstrating that N-acetyl-cysteine facilitated IL-1β-induced nitric oxide synthase (iNOS) expression in rat vascular smooth muscles cells through a reduction/oxidation-related mechanism involving potentiation of cytokine activation of the p44/42 MAPK signaling pathway. Another explanation was given by Huang and colleagues[30], who reported that ascorbic acid enhanced endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin BH_4 content. Concerning the regulation of COX activity by antioxidants, our study was in disagreement with other studies which reported that known antioxidants, such as butylated hydroxyanisol or diphenylamine, were also potent inhibitors of arachidonic acid metabolism[31].

ACECLO is a NSAID of the phenylacetic acid class, commonly used in the treatment of arthritis. Interestingly, the mechanism of action of ACECLO is multifactorial. Indeed, this NSAID acts not only by inhibiting preferentially the COX-2 activity but also by decreasing proinflammatory cytokines synthesis and by scavenging reactive oxygen species[32]. At $1 \cdot 10^{-5}$ M, ACECLO decreased luminol-enhanced chemiluminescence generated by PMA-activated neutrophils and accelerated the decomposition of $ONOO^{-}$, suggesting a potential capacity of this drug to scavenge $ONOO^{-}$. In contrast, and by comparison with other classical NSAID, ACECLO did not reduced lipid peroxydation induced by Fe^{2+}/ascorbate system and did not significantly reduced HOCl induced chemiluminescence. Furthermore, we have recently demonstrated that, at therapeutic concentration (ranged among 6 to 10 10^{-6} M) ACECLO did not react with $^{\cdot}OH$ or $O_2^{\cdot-}$ (submitted data). Taken together, these findings demonstrated that ACECLO preferentially inhibited $ONOO^{-}$ but not $O_2^{\cdot-}$, $^{\cdot}OH$ or HOCl. Considering these particular antioxidant properties, we have tested this drug on the expression of COX-2, IL-1β and iNOS genes, and on the synthesis of PGE_2 and NO. Interestingly, ACECLO fully blocked PGE_2 synthesis without affecting COX-2 gene expression. Furthermore, we have previously reported that ACECLO strongly inhibited COX-2 activity ($IC_{50}=8 \cdot 10^{-7}$ M) in the whole blood test but showed an anecdotal effect on purified cyclo-oxygenases[33]. These observations suggested that ACECLO probably exerts its inhibitory action on PGE_2 synthesis at the post-transcriptional level but not by a direct interaction with the catalytic site of COX.

Taken together, these data clearly demonstrate that NO down-regulates PGE_2 synthesis at the post-transcriptional

level and that antioxidants, such as N-acetyl-cysteine, increase NO synthase activity and expression. These findings suggest that antioxidant therapy could have contradictory effects as a function of the molecule administered, especially the oxygen radical targeted. To block NO could result in an increase of PGE_2 production, which was clearly identified as an inflammatory and catabolic factor. Increasing the antioxidant status of the cell could induce an overproduction of NO.

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