Expression of TGF-βs and their receptors is differentially modulated by reactive oxygen species and nitric oxide in human articular chondrocytes

N. Ayache*, K. Boumediene*, M. Mathy-Hartert†, J.-Y. Reginster†, Y. Henrotin† and J.-P. Pujol*

*Laboratory of Connective Tissue Biochemistry, University of Caen, France
†Bone and Cartilage Metabolism Research Unit, CHU Sart-Tilman, Liege, Belgium

Summary

Objectives: To study the effects exerted by two antioxidants, N-nomethyl-L-arginine (L-NMMA), as an inhibitor of nitric oxide (NO) synthesis, and N-acetylcyesteine (NAC), reactive oxygen species (ROS) scavenger, on the expression of the major growth factor involved in cartilage repair, TGF-β, under the three isoforms β1, β2 and β3, and the receptors I and II of this factor, using lipopolysaccharide (LPS)-treated human chondrocytes in culture.

Methods: Suspension cultures of human chondrocytes derived from the knee of osteoarthritic patients were treated for 48 h with lipopolysaccharide (LPS) (10 µg/ml), L-NMMA (0.5 mM) or NAC (1 mM). Nitrite levels were assayed on the culture media using the Griess spectrophotometric method. After total RNA extraction, the expression of inducible NO synthase (iNOS), TGF-β1, TGF-β2, TGF-β3, TGF-β receptors I and II, was determined by semi-quantitative polymerase chain-reaction (RT-PCR).

Results: LPS induced a dramatic increase of both NO production and iNOS mRNA level. The addition of L-NMMA (0.5 mM) abolished NO production without affecting iNOS mRNA levels. In contrast NAC (1 mM) strongly synergized with LPS to stimulate NO synthesis. LPS treatment did not significantly alter TGF-β1 expression whereas L-NMMA inhibited its production. TGF-β2 mRNA level was decreased by LPS and was not changed in the presence of L-NMMA. On the other hand, NAC was capable of counteracting the LPS-induced inhibition of TGF-β2 expression. TGF-β3 mRNA level was markedly reduced by LPS alone, or with both L-NMMA and NAC. Finally, the expression of TGF-βRII was slightly increased in the presence of combined LPS and L-NMMA or NAC whereas that of TGF-βRII was reduced in the same conditions.

Conclusions: The modulation of TGF-β system was found to be differentially controlled by NO and ROS productions. Indeed, the control exerted on TGF-β expression varied according to the isoform: TGF-β1 mRNA level depends on NO whereas that of TGF-β2 is regulated by ROS and TGF-β3 seems to be unaffected by both of them. The expression of TGF-β receptors appeared to be modulated by NO and ROS levels. The relevance of the present findings to osteoarthritis (OA) physiopathology and the potential use of antioxidant therapy to treat this disease are discussed. © 2001 OsteoArthritis Research Society International. Published by Elsevier Science Ltd. All rights reserved.

Key words: Articulate chondrocytes, Nitric acid, Reactive oxygen species, Transforming growth factor-βs

Introduction

Progressive loss of cartilage is the characteristic feature of joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). In late stages of the pathology, this may lead to joint failure and implantation of prothesis remains today the only clinical treatment possible. Therefore, chondroprotection of the cartilage is one of the most important and attractive goals to achieve in this medical field.

It is now recognized that the homeostasis of cartilage results from the balance between its anabolic and its catabolic rates. Depletion of cartilage occurs in OA through both breakdown of the matrix and reduced synthesis of its macromolecular components. Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) have been shown to be implicated in these mechanisms since they are present in excess in affected joints and are capable of degrading the cartilaginous matrix while inhibiting its synthesis. On the other hand, local growth factors produced by chondrocytes, such as transforming growth factor-β (TGF-β), can counteract the deleterious effects of IL-1 and TNF-α through stimulation of matrix synthesis and inhibition of metalloprotease expression. TGF-β, which is expressed under its three isoforms (β1, β2 and β3) in cartilage, appears as one of the major element in the repair potential of the tissue. The dramatic decrease of TGF-β receptor II expression by chondrocytes at the stage of fibrillated cartilage in the experimental rabbit OA model and the appearance of OA-like lesions in transgenic mice expressing nonfunctional receptor II strongly suggest that the TGF-β system takes a great part in the repair potential of articular cartilage. Thus, the mechanisms which control the expression of TGF-β systems in articular chondrocytes submitted to physiological or pathological situations merit particular attention, in view of future therapeutic approaches aimed at chondroprotection.

In this regard, it is of great interest to determine what could be the effects exerted by nitric oxide (NO) and reactive oxygen species (ROS) on the expression of TGF-β
and its receptors by articular chondrocytes. Nitric oxide is synthesized during metabolism of L-arginine to NO₂⁻ or NO⁻15,16 and is produced in large amounts by articular chondrocytes in response to IL-117–19. Several reports have demonstrated that NO was involved, at least partially, in the IL-1-induced suppressive effect on cartilage proteoglycan synthesis through inhibition of the core protein synthesis20–23. It also mediates the IL-1 stimulatory effect exerted on gene expression of matrix metalloproteinases in rabbit articular chondrocytes24. Indeed, it has been reported that the concentration of nitric oxide, which represents the local NO production, is increased in synovial fluid and sera from patients with inflammatory or degenerative diseases such as OA and RA25,26. Accumulating evidence also suggests that ROS are not only injurious by-products of cellular metabolism but also essential participants in cell signaling and regulation in most cell types, including articular chondrocytes (review in 27). It is known that ROS are active on various components of the cartilage matrix, such as hyaluronic acid20,22,23,26,29,30. They may also influence articular chondrocyte growth31, induce membrane damage32 and reduce their metabolic pathways for energy production32,33. It has been revealed that free radicals are involved in arthritic conditions33. Among the ROS produced by chondrocytes, hydrogen peroxide is increased by cytokines such as IL-1, TNF-α and IFN-γ26–41 and the chondrocytes have been shown to be damaged by hydrogen peroxide42. Superoxide (O₂⁻) and nitric oxide (NO) can react to form the most potent peroxynitrite anion43. Potentially toxic levels of peroxynitrite can be achieved in joint tissues under conditions in which superoxide (O₂⁻) and NO production are simultaneously stimulated by IL-113. As far as we know, the hypothesis that NO and ROS might participate in the control mechanisms of cytokine and growth factor expression by articular chondrocytes has not yet deserved much attention and only little information is available about the sensitivity of TGF-β system to these active oxygen species44. The hypothesis gains strong support from the finding that the activity of nuclear transcription factors, such as NF-κB and AP-1, which are involved in the transcription of several genes implicated in arthritic situations, is differentially modulated by ROS45. In order to get insights into the roles played by these species in the physiological and pathobiological metabolism of cartilage cells, we have investigated here the expression levels of TGF-β isoforms and TGF-β receptors I and II in human articular chondrocytes under LPS activation and in the presence of inhibitors of NO and ROS.

III and two cartilage samples had lesion of grade I. Each culture was run with chondrocytes from a single donor. Chondrocytes were released by sequential enzymatic digestion, using hyaluronidase, pronase and collagenase (10 ml enzyme solution per 3 g of cartilage). The cartilage fragments were first treated (30 min, 37°C, 200 rpm) with hyaluronidase (Sigma-Aldrich, Bornem, Belgium) previously dissolved (0.5 mg/ml) in Dulbecco’s modified Eagle’s Medium supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (DMEM; Biowhittaker, Brussels, Belgium). The fragments were then treated (1 h, 37°C, 200 rpm) with pronase solution (Merck-Belgolabo, Overijse, Belgium) (1 mg/ml in DMEM). Finally, they were incubated (20 h, 37°C, 200 rpm) with collagenase solution (Sigma-Aldrich, Bornem, Belgium) (1 mg/ml in DMEM containing 1% Ultrroser G (Life Technologies, Meribèke, Belgium). The cells were incubated at the density of 1.10⁵/ml in 1 ml of 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 0.625 mg of insulin, 0.625 mg of transferrin, 0.625 μg of sodium selenite, 125 mg of bovine serum albumin (BSA) and 0.535 mg of linoleic acid/ml. The cells were then maintained in culture medium for 48 h in order to eliminate the potential influence of drug treatment previously taken by the donors. They were then seeded in 15-ml polypropylene Falcon tubes and maintained under agitation on a gyratory shaker (100 rpm) in a 95% air/5% CO₂ atmosphere. Chondrocytes were cultured for 48 h in the absence or in the presence of LPS (10 μg/ml, serotype 026:66; Sigma-Aldrich, Bornem, Belgium) and with or without L-NMMA (0.5 mM; Calbiochem, San Diego, U.S.A.) or NAC (1 mM; Sigma-Aldrich, Bornem, Belgium). Cells and supernatant were then separated by centrifugation (1000 rpm; 5 min).

**NITRIC OXIDE ASSAY**

NO production was determined by quantifying nitrite in conditioned medium using spectrophotometric method based on the Griess reaction. Briefly, 150 μl of conditioned culture medium or sodium nitrite (NaNO₂) standard dilutions were mixed with 100 μl of Griess reagent (0.5% sulfanilamide, 0.05% naphthylethylenediamine dihydrochloride, 2.5% H₃PO₄) 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, Bruxelles, Belgium). The absorption was recorded at 540 nm.

**Materials and methods**

**CULTURE OF HUMAN CHONDROCYTES**

Cartilage samples 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. They were dissected from superficial and medium layers, avoiding the calcified zone. Femoral and patellar articular surfaces were evaluated for the severity of the macroscopic lesions, using a personal scale of four grades: 0, normal white cartilage on all area 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 surfaces. Three out of the five cartilage samples collected showed lesion of grade...
40 μl of PCR reaction with 0.2 μl of Taq polymerase (15 units/μl), 1 μl of dNTPs (10 mM each), 1 μl of 50 mM MgCl₂ and 20 μM of each primer (Life Technologies, Gibco, BRL). The following amplification protocol was used: 1 min at 95°C; 1 min at 55°C; 1 min at 72°C. The primer sequences are listed in Table I. PCR amplification for Glyceraldehyde 3-Phosphate dehydrogenase (GAPDH) was performed as an internal control for the relative amounts of templates. PCR products were analysed in 2% agarose gels, after staining with ethidium bromide. Densities of the band were used to quantitate the messages values corresponding to the linear part of the amplification expression of messengers studied was calculated as relative to GAPDH. Densities of the band were used to quantitate the messages values, contrasting with the findings on NO production [Fig. 1(b)]. These data support the hypothesis that the co-operation between NAC and LPS in NO formation is exerted on the iNOS enzyme activity rather than on the transcriptional level of the gene.

NO AND ROS DIFFERENTIALLY CONTROL THE EXPRESSION OF TGF-β1

TGF-β1s are known to counteract several deleterious effects of IL-1 in several tissues, including cartilage. Therefore, it was of interest to estimate the expression level of the three representative members of this family in articular chondrocytes upon LPS-induced production of NO and ROS. As can be seen in Fig. 2(a), the steady-state levels of TGF-β1 mRNA were significantly decreased in the presence of L-NMMA in both control and LPS-treated cultures, indicating that NO positively controls the transcriptional activity of TGF-β1 gene. In contrast, no significant effect was observed when cells were exposed to NAC, an inhibitor of ROS production. This finding suggests that the maintenance of basal TGF-β1 expression is NO-dependent, at least in our experimental conditions. TGF-β2 mRNA levels were found to be differentially modulated, as LPS alone could induce a significant reduction of the messenger which was not further changed in the presence of NO inhibitor [Fig. 2(b)]. On the other hand, ROS inhibition by NAC fully reversed the LPS inhibitory effect. This apparent co-operation of LPS and NAC suggests that ROS are involved in LPS-induced inhibition of TGF-β2 expression. In what concerns TGF-β3, its expression was found to be markedly depressed in cultures exposed to LPS but, in contrast to the TGF-β1 and 2 isoforms, no effect of both L-NMMA and NAC was observed [Fig. 2(c)]. These data clearly indicate that the down-regulation of TGF-β3 expression by LPS is likely to be independent of NO and ROS levels.

Results

BLOCKADE OF ROS FORMATION STIMULATES LPS-INDUCED NO PRODUCTION IN ARTICULAR CHONDROCYTES

Human articular chondrocytes cultured in suspension produced very low levels of NO, in the present experimental conditions [Fig. 1(a)]. However, in the presence of LPS (10 μg/ml) for 48 h, the cells were strongly stimulated and released high levels of nitric oxide (about 14 times more). As expected, this effect was completely abolished in the presence of L-NMMA (0.5 mM), an inhibitor of iNOS enzyme activity. In contrast, NAC (1 mM) which is known as a molecule neutralizing ROS formation, was capable of stimulating NO production in both basal and LPS-treatment conditions. Furthermore, the results constantly showed a synergistic effect of this compound on LPS action [almost double value; compare bar 6 to bar 2 in Fig. 1(a)], suggesting that blockade of ROS formation may reinforce the activity of the NO metabolic pathway.

The effect of LPS on NO production was mirrored at the level of iNOS mRNA steady-state levels which were increased by approximately 8 folds [Fig. 1(b)]. This action was not modified in the presence of L-NMMA, confirming that the inhibitor has no effect on transcriptional activity of iNOS gene. Interestingly enough, addition of NAC to the cultures induced an elevation of iNOS messenger amount comparable to that elicited by LPS treatment. However, there was no apparent synergism with LPS at the level of mRNA values, contrasting with the findings on NO production [Fig. 1(b)]. These data support the hypothesis that the co-operation between NAC and LPS in NO formation is exerted on the iNOS enzyme activity rather than on the transcriptional level of the gene.

Table I

<table>
<thead>
<tr>
<th>Nucleotide sequences of RT-PCR primer pairs (5′—3′ orientation)</th>
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<tbody>
<tr>
<td>TGF-β1 Sense</td>
</tr>
<tr>
<td>Antisense</td>
</tr>
<tr>
<td>TGF-β2 Sense</td>
</tr>
<tr>
<td>Antisense</td>
</tr>
<tr>
<td>TGF-β3 Sense</td>
</tr>
<tr>
<td>Antisense</td>
</tr>
<tr>
<td>TypeⅠ TGF-β receptor Sense</td>
</tr>
<tr>
<td>Antisense</td>
</tr>
<tr>
<td>TypeⅡ TGF-β receptor Sense</td>
</tr>
<tr>
<td>Antisense</td>
</tr>
<tr>
<td>GAPDH Sense</td>
</tr>
<tr>
<td>Antisense</td>
</tr>
</tbody>
</table>

Despite the fact that LPS treatment did not induce significant variation in the steady-state levels of mRNA encoding TGF-β1 receptors I and II, we found a slight elevation for TGF-β1 receptor I and a clear reduction for receptor II mRNA in the presence of either L-NMMA or NAC [Fig. 3(a),(b)]. In the case of TGF-β receptor II, it is worth mentioning that the inhibitors are already effective in the absence of LPS [Fig. 3(b)]. These data support the hypothesis that the presence of some level of NO and ROS may partially contribute to stimulate the expression of TGF-β receptor II.

Discussion

We used a lipopolysaccharide (LPS) model of chondrocyte activation to determine the respective role of NO and ROS on the expression of TGF-β1 and its receptors. Previous studies have shown that LPS can induce the production by chondrocytes of NO and ROS including H₂O₂, O₂ and “OH. A first basic information can be drawn from
our results: blockade of chondrocyte-derived ROS exerts a positive effect on NO production, already in the absence of LPS activation. This effect was shown to reinforce the stimulative action of LPS on NO synthesis, probably by mechanisms which not only involve transcriptional control but other steps, including possible increase of the enzyme activity itself, since no significant change in iNOS mRNA steady-state levels was found between LPS-activated chondrocytes in the absence or presence of the antioxidant N-acetyl-cysteine (NAC). It is worth mentioning that similar findings have been reported in the case of IL-1 activation, showing that NAC facilitated the cytokine-induced iNOS expression in rat vascular smooth muscle through a reduction/oxidation mechanism potentiating the p44/42 MAPK signalling pathway. As an explanation of this interaction between ROS and NO pathways, it can be suggested that ROS produced intracellularly in response to LPS may react with NO to form peroxynitrite (ONOO−), which is considered as a potential enzyme inactivator. In the absence of ROS, this negative effect could be cancelled, resulting in iNOS induction. However, the mechanisms underlying these findings remain to be experimentally elucidated. Since the effect observed here was obtained with the antioxidant NAC, it would be particularly interesting to determine if it is specific to this molecule or if similar results could be obtained with other reductant compounds. Indeed, NAC has been shown to suppress nerve growth factor (NGF)-induced activation of AP-1 transcription factor by uncoupling the upstream signal transduction from RAS to the MAP kinase cascade in PC12 cells and the inhibitory effect of NAC in that case is unlikely to be due to the suppression of ROS, because...
NGF does not stimulate the production of ROS in PC12 cells. Whatever mechanisms are responsible for the induction of iNOS expression in chondrocytes under ROS blockade, this effect must be taken into consideration when therapeutic approaches of OA based on ROS inhibition are envisaged.

Contrasting with the previously reported inhibition exerted on TGF-β1 expression by IL-1-induced NO\textsuperscript{44}, LPS-induced NO in our model does not produce the same effect on TGF-β1 mRNA levels. First, LPS treatment did not significantly alter TGF-β1 mRNA level. Secondly, the NO inhibitor L-NMMA caused a decrease of these levels in the

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Fig. 2. Effect of L-NMMA and NAC on the expression of the three isoforms of TGF-β in LPS-treated human chondrocytes. RT-PCR was performed in the same conditions as in Fig. 1 and PCR products were analysed in a 2% gel agarose.
absence as well as in the presence of LPS activation, suggesting that in our model some level of NO is required for maintenance of TGF-β1 expression. It is not surprising that the effects of NO observed in our model, using LPS as an activator of chondrocytes instead of IL-1, could be different, since it is likely that the action of intracellular NO is dependent on the cross-talks it may establish with the other signaling pathways elicited either by LPS or IL-1. This interpretation is also supported by the fact that NO produced by addition of exogeneous donor such as SNAP (S-nitroso-N-acetylpenicillamide) did not produce the same effect as IL-1 in the study by Studer et al.\textsuperscript{44}. TGF-β2 expression in LPS-treated chondrocytes was found here to be differentially controlled, compared to TGF-β1. Activation of the cells resulted in a significant reduction of TGF-β2 mRNA steady-state levels which was not dependent on NO. On the other hand, the expression of TGF-β2 was clearly enhanced when the production of other ROS was suppressed, indicating that some ROS normally exert a down-regulation on TGF-β2 expression. Finally, TGF-β3

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**Table II**

*Summary of the potential effects of NO and ROS on the expression of TGF-β and its receptors in cultured human chondrocytes*

<table>
<thead>
<tr>
<th>LPS (10 µg/ml)</th>
<th>Blockade of NO production by L-NMMA (0.5 mM)</th>
<th>Blockade of ROS production by NAC (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>=</td>
<td>= Influence of NO</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>_</td>
<td>_ Influence of ROS</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>_</td>
<td>_ No apparent influence</td>
</tr>
<tr>
<td>TGF-β RI</td>
<td>=</td>
<td>_ Influence of NO and ROS</td>
</tr>
<tr>
<td>TGF-β RII</td>
<td>=</td>
<td>_ Influence of NO and ROS</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of L-NMMA and NAC on the expression of TGF-β receptors in LPS-treated human chondrocytes. RT-PCR was performed in the same conditions as in Fig. 1 and PCR products analysed in the same conditions as for other messengers.
expression was found to be substantially depressed by LPS but no dependence towards NO or ROS production could be established in the present study. A definition of how NO and ROS could differentially influence the expression of the three TGF-β isoforms in these experiments remains speculative because the mechanisms that are involved in the control of TGF-β gene promoters are not completely understood. However, it is known that TGF-β1 promoter structure differs from that of the two other isoforms by several characteristics. It contains regulatory sequences, such as AP-1 binding sites and several GC-rich zones which can bind Sp-1-like transcription factors, but lacks TATA and CAAT boxes48. On the other hand, TGF-β2 and TGF-β3 promoters present respectively two and one TATA boxes. They contain CRE (cAMP responsive elements)49,50 but whereas an AP-1 site is present in TGF-β2 gene, it is not functional, and TGF-β3 promoter does not contain this later. Sp-1 seems to be implicated in TGF-β3 but not TGF-β2 expression51. The specific features of TGF-β1 promoter probably explain that this isoform has been given the most prominent role in the early events of tissue remodeling, such as wound healing, where its particular sensitivity to cytokine regulation has been demonstrated. In this regard, it is interesting to note that in our model NO was found to be required for its basal expression. We may speculate that the NO produced during the early lesion of cartilage could induce a positive feed-back on TGF-β1 expression as a beneficial repair reaction. Despite the fact that TGF-β2 is weakly expressed in articular cartilage52, it may have some important role in osteoarthritic tissue. However, it is difficult to determine what could be the importance of the ROS-induced negative control observed here on TGF-β2 isoform in the context of cartilage physiopathology. In what concerns TGF-β3, which is expressed at relative high amount in cartilage, it is clear that activation of chondrocytes by LPS results in reduction of this isoform expression but the mechanism is not dependent on NO and ROS production.

Interestingly, we observed that the expression of TGF-β receptor II was positively controlled by NO and ROS levels. We have previously reported that the response of cultured chondrocytes to TGF-β was dependent on the ratio between TGF-β receptor I and receptor II levels, this later varying according to the cell proliferation state11. Furthermore, we have demonstrated in the OA rabbit model that the expression of TGF-β receptor II, the one which binds the ligand, disappears almost totally in heavily degraded cartilage, suggesting that the chondrocytes become insensitive to the growth factor and then cannot counteract deleterious effects of IL-112. Therefore, NO and ROS up-regulation of TGF-β receptor II expression be considered as a beneficial feed-back mechanism elicited by the deleterious cytokines, such as IL-1 and TNF-α, in the early steps of OA.

In summary, our findings suggest that the cellular redox state regulates the cellular responses of chondrocytes in concert with extracellular stimuli. It may be a significant factor in altering the respective expression of the crucial TGF-β growth factor system, which is known to favor cartilage repair. For example, excessive production of NO and ROS observed in osteoarthritic joint tissues may serve as a feed-back element to reduce IL-1 effects, through stimulating TGF-β receptor II expression, in a sort of ‘yin yang’ mechanism. However, it is likely that the modulation of these different genes is greatly dependent on the dosage of the redox signal, which in turn is supposed to vary during the course of the OA process. Therefore, both in vitro and in vivo studies should be combined to investigate the dual redox regulation of cellular signaling pathways in chondrocytes and the cross talk between cellular redox and signaling. Because of the potential feed-back loops between NO, ROS and cytokine/growth factor expression in cartilage cells, it is so far difficult to conclude on the possible ways to manipulate these actors for gaining beneficial improvement of OA treatment.

References


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