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Onga, Takafumi
Saura, Ryuichi
Mizuno, Kosaku
Itoh, Hiroshi

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EFFECT OF PROSTAGLANDIN E_2 ON NITRIC OXIDE SYNTHESIS IN ARTICULAR CHONDROCYTES

Takafumi ONGA*,**, Ryuichi SAURA*, Kosaku MIZUNO*,
and Hiroshi ITOH**

*Department of Orthopaedic Surgery, Kobe University School of Medicine
**First Division, Department of Pathology, Kobe University School of Medicine

KEY WORDS

articular cartilage; chondrocyte; nitric oxide; prostaglandin E_2; osteoarthritis

ABSTRACT

Both nitric oxide (NO) and prostaglandin E_2 (PGE_2) are known to play an important role in cartilage metabolism. The present study investigated the novel intercellular mechanism of inducible NO synthase (iNOS) induction mediated by PGE_2 in articular chondrocytes. Bovine articular chondrocytes were stimulated by cyclic adenosine monophosphate (cAMP) elevating agents like PGE_2 in the presence of interleukin-1α (IL-1α). NO generation was measured by using the Griess reaction. Inducible NOS

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mRNA was semi-quantitated by reverse transcription-polymerase chain reaction (RT-PCR). While little NO was released from articular chondrocytes in the presence of PGE_2 or direct adenylate cyclase activator such as forskolin, synergistic augmentation of NO generation was observed when chondrocytes were stimulated by PGE_2 or forskolin in combination with IL-1α. Further expression of iNOS mRNA by stimulation of PGE_2 in the presence of IL-1α simultaneously was also detected by RT-PCR in comparison with the mRNA induction by IL-1α stimulation alone. These results indicated that PGE_2 might modulate the articular cartilage metabolism by augmentation of chondrocyte NO synthesis in inflammatory process through cAMP-protein kinase A system.

INTRODUCTION

Increased levels of nitric oxide (NO) and prostaglandin E_2 (PGE_2) are presented in the synovial fluid from patients with rheumatoid arthritis (RA) or osteoarthritis (OA).[4, 8] Recently both NO and PGE_2 have been implicated to play an important role in cartilage metabolism and in pathophysiology of inflammatory joint diseases such as RA or OA.

Nitric oxide is a free radical gas mediator with a short biological half-life, which had been originally shown to be the endothelium-derived relaxing factor (EDRF).[23] In articular joints, mesenchymal cells are reported to release NO such as macrophages and synovial cells. Chondrocytes were also demonstrated to produce a large amount of NO by inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα). The evidences of either pro- or anti-inflammatory effects of NO are reported.[30] For instance, NO suppresses the chondrocyte proliferation through the induction of apoptosis in vitro,[2, 3] which may cause cartilage matrix degradation, while there are anti-inflammatory effects including the inhibition of neutrophil adhesion to endothelial cells[16] and superoxide production by neutrophils.[28]

Nitric oxide production is regulated by NO synthase (NOS). There are three types of isoform, two constitutive forms of NOS (ecNOS, nNOS) and inducible type of NOS (iNOS).[7] Once iNOS is promoted by the stimulation of proinflammatory cytokines or lipopolysaccharide (LPS),[29] large amounts of NO, which have been implicated in the control of host defense mechanisms[31] and immunoregulation,[35] are produced. Therefore, this type of NOS is considered to play an important role in articular cartilage metabolism in inflammatory condition.

Prostaglandin, a product of arachidonic acid synthesized by cyclooxygenase (COX), is also reported to be one of the important regulators for the cellular functions in a variety type of tissues, including bone and cartilage. Therefore, high concentration of PGE_2 detected in joint fluid of RA patients,[34] is suggested to play a significant role in the pathogenesis of the joint disorders.[5]

Cyclooxygenase-2 is the inducible form of COX in inflammatory condition, while COX-1 is expressed constitutively in order to regulate the cellular function physiologically. In terms of the interaction of NO and PGE_2, the relationship of iNOS and COX-2 has not been well studied. Nitric oxide has been shown to stimulate COX-2 activity directly.[2, 6, 11, 14, 25, 26, 28, 32] Reversely, PG is reported to modulate the induction of iNOS activity.[22, 33] The purpose of this study is to examine the effects of PGE_2 on chondrocyte NO synthesis and to investigate the intracellular signaling pathway of iNOS induction in articular chondrocytes through cAMP-A kinase system.

MATERIALS AND METHODS

Cell culture

Bovine articular cartilage was isolated from carpo-metatarsal joints of adult cows within 2 hours after slaughter and cut into thin sections. Articular chondrocytes were

obtained from these tissues by collagenase digestion as described with some modification.[27] Briefly, tissues was minced and treated with 10 mg/ml of collagenase (Sigma, St. Louis, MO) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) for 6 hours at 37°C. The isolated chondrocytes were harvested by filtration through gauze to remove tissue fragments, followed by centrifugation (2000rpm for 10 min.). The cells were resuspended in DMEM, supplemented with 10% heat-inactivated fetal calf serum (FCS; Bioserum, Melbourne, Victolia, Australia) and antibiotics (20 units/ml penicillin and 100 μg/ml streptomycin). This complete medium was used in all chondrocyte assays unless stated otherwise. The isolated cells, which were routinely checked for rounded (cobble stone) morphology, consistent with the differentiated phenotype,[17, 18] were cultured in a monolayer fashion, then subjected to experiments described below. Articular chondrocytes in the complete medium were allowed to attach in every well of 24-multiwell flat bottomed culture plates (Corning Glass Works, Corning, NY) at the cell density of 5.0×10^5 cells/well. Then chondrocytes were cultured for 24 hours in the serum free DMEM prior to experiments. After the chondrocytes were stimulated in the presence of the stimuli such as PGE_2 or forskolin (Sigma) with or without IL-1α (Genzyme, Boston, MA), the sample aliquots were assayed as follows.

NO measurement

Nitric oxide generation of articular chondrocyte was detected by measurement of nitrite accumulation in the culture supernatants by addition of Griess reagent.[9] Briefly, the sample aliquots were incubated with the reaction reagents composed of 1 % sulfanilamide, 0.1 % N-1-naphthylethylenediamide dihydrochloride in 25 % H_3PO_4 at room temperature for 5 min. Nitrite concentration was measured by a spectrophotometer at 550 nm using a standard curve calibrated by sodium nitrite.

Preparation of RNA (mRNA) and RT-PCR

For the detection of iNOS mRNA expression of articular chondrocytes, 1.0×10^6 cells were inoculated into the culture dish in 35 mm in diameter and total RNA was directly isolated from the cell monolayers using RNA zol^{TM}B (Tel-test, Friendswood, TX) according to the manufacturer's instructions. RT-PCR was performed using a protocol as reported previously with some modifications. [1, 12] One μg of total RNA was reverse-transcribed in 40 μl containing 5 mM $MgCl_2$, 10×PCR buffer II, 1 mM dNTPs, 50 units of MuLV Reverse Transcriptase (Perkin Elmer, Foster City, CA) for 60 minutes at 42°C. PCR was performed with 5 μl from the total 40 μl of RT product with 2 units of AmpliTaq DNA polymerase (Perkin Elmer), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, and 0.2 μM of each primer. The primers specific for bovine iNOS were 5'-GTGGAAGCAGTAACAAAGGAG-3' (sense) and 5'-CTGCCATCTGGCATCT GGTAG-3' (antisense). These primers amplified a 332 basepair PCR product. The primers specific for human GAPDH were 5'-GTGAAGGTCGGAGTCAACG-3' (sense) and 5'-GAGATGATGACCCTTTTGGC-3' (antisense). These primers amplified a 356 base pair product. The following amplification protocol was used for bovine iNOS: 3 minutes at 94°C, 1 minute at 94°C (denature), 2 minutes at 55°C (anneal), 3 minutes at 72°C (extend), for 35 cycles and human GAPDH: 30 minutes at 60°C (reverse transcription), 3 minutes at 94°C, 1 minute at 94°C (denature), 2 minutes at 55°C (anneal), 3 minutes at 72°C (extend), for 28 cycles.

A fraction of each sample product was analyzed by electrophoresis in a 1.5 % agarose gel. DNA was stained by ethidium bromide and visualized with an ultraviolet illuminator. Photographs of the gels were digitized by a scanner and then analyzed with the public-domain NIH Image software as previously described.[13]

Statistical analysis

Results are expressed as mean ± SEM. For all results shown at least two separate experiments with cells from different tissue were performed. Statistical analysis was performed with Student's t-test. p values less than 0.05 were considered statistically significant.

RESULTS

Nitric oxide generation from the articular chondrocyte stimulated by proinflammatory cytokines and effect of cyclooxygenase inhibitor on chondrocyte nitric oxide synthesis.

NO production of articular chondrocytes stimulated by IL-1α was observed in a dose- and time-dependent fashion (Figure 1). Nitrite concentration was elevated significantly at the presence of 10 U/ml of human recombinant IL-1α. Both IL-1α and human recombinant TNFα stimulate NO release from bovine chondrocytes. However, human recombinant IL-1β failed to stimulate NO release of bovine chondrocytes. This might be due to the difference of IL-1 receptor between these species. These results suggest that articular chondrocytes generate NO in the presence of proinflammatory cytokines.

This IL-1α -induced NO generation was partially suppressed by addition of a direct COX-2-selective inhibitor, 6-methoxy-2-naphtyl acetate (6-MNA) (Cayman chemical company, Ann Arbor, MI) in a dose-dependent manner. Significant suppression was observed in addition of 1 μM of 6-MNA (Figure 2). These data implicate that prostaglandins may have an important role on NO synthesis in articular chondrocytes induced by proinflammatory cytokines. We have, therefore, examined the effects of PG on chondrocyte NO synthesis. When only PGE_2 was added exogenously in the chondrocyte culture, little effect on chondrocyte NO synthesis was presented. However, when chondrocytes were treated with PGE_2 in the presence of IL-1α simultaneously,

chondrocyte NO release was augmented in dependent of the PGE_2 concentration (Figure 3). PGE_2 are reported to activate adenylate cyclase, which synthesizes cAMP as intracellular signal transduction.[20] Then the effect of forskolin, a direct activator of adenylate cyclase on chondrocyte NO generation was examined. The forskolin has augmented NO release from the articular chondrocytes in the presence of IL-1α, while forskolin alone failed to enhance chondrocyte NO generation (Figure 4).

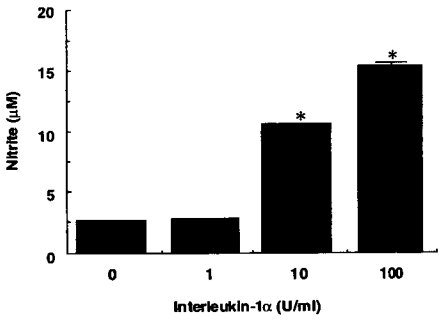

Fig. 1. Nitric oxide (NO) generation from articular chondrocytes in response to increased concentration of interleukin-1α (48 h). Chondrocytes were cultured at the cell density of 5.0 x 10^5 cells/well for 48 hours with culture medium alone or with interleukin-1α (IL-1α). NO generation was detected by measurement of nitrite accumulation in the culture supernatants by addition of Griess reagent. Data represent the means±SEM (n =3). *p < 0.05.

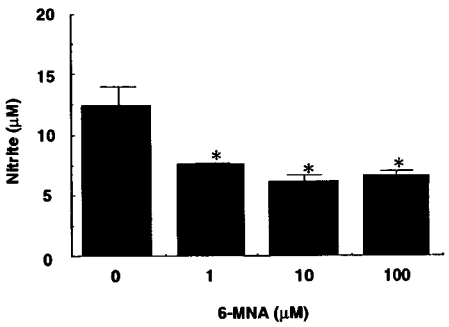

Fig. 2. Effect of COX-2 selective inhibitor, 6-methoxy-2-naphtyl acetate (6-MNA) on chondrocytes NO synthesis. Chondrocytes were cultured at the cell density of 5.0 x 10^5 cells/well for 48 hours with various concentration of 6-MNA in the presence of 10 U/ml of IL-1α. NO generation was detected by measurement of nitrite accumulation in the culture supernatants by addition of Griess reagent. Data represent the means±SEM (n =3). *p < 0.05.

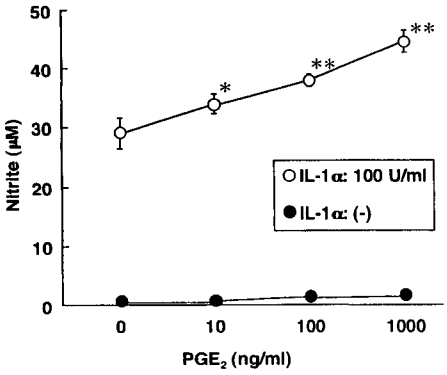

Fig. 3. Effect of prostaglandin (PG) on chondrocyte NO synthesis. Chondrocytes were cultured at the cell density of 5.0 x 10^5 cells/well for 48 hours with various concentration of PGE_2 in the presence of 100 U/ml of IL-1α (○) and in the absence of IL-1α (●). NO generation was detected by measurement of nitrite accumulation in the culture supernatants by addition of Griess reagent. Data represent the means±SEM (n =3). *p < 0.05, ** p < 0.01.

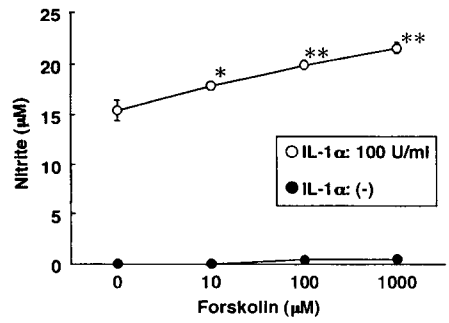

Fig. 4. Augmentation of IL-1α induced NO synthesis by direct adenylate cyclic activator, forskolin. Chondrocytes were cultured at the cell density of 5.0 x 10^5 cells/well for 48 hours with various concentration of forskolin in the presence of 100 U/ml of IL-1α (○) and in the absence of IL-1α (●). NO generation was detected by measurement of nitrite accumulation in the culture supernatants by addition of Griess reagent. Data represent the means±SEM (n =3). *p < 0.05, ** p < 0.01.

Effect of either prostaglandin or adenylate cyclase activator on inducible Nitric oxide synthase mRNA expression in the articular chondrocyte.

In order to detect the expression of the iNOS mRNA of bovine articular chondrocytes, RT-PCR was performed. It is revealed that articular chondrocytes have little expressed iNOS mRNA without stimulation. Though IL-1α treatment has enhanced the expression of small amount of iNOS mRNA, marked augmentation of iNOS mRNA expression was observed when articular chondrocytes were treated with PGE_2 or forskolin in the presence of IL-1α simultaneously (Figure 5). Prostaglandin E_2 or

forskolin was failed to induce iNOS mRNA. These results suggest that synergistic enhancement of NO production by cAMP elevating agents occur at the transcriptional level.

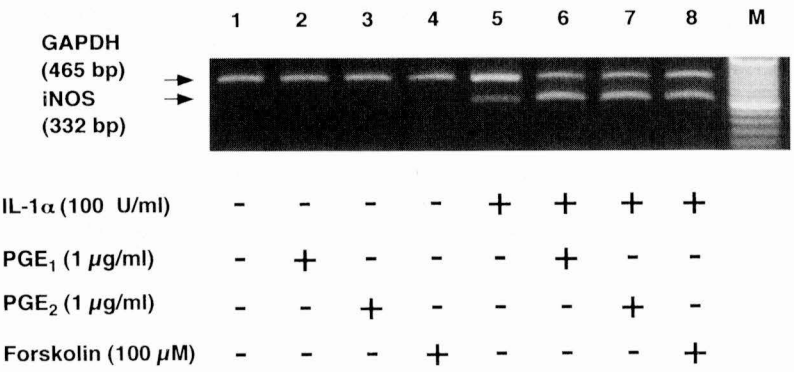

Fig. 5. Expression of iNOS and GAPDH mRNA in cultured articular chondrocytes with various stimuli. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with primers for iNOS and GAPDH. Lane 1, unstimulated. Lane 2, treated with 1 μg/ml of PGE_1 without IL-1α. Lane 3, treated with 1 μg/ml of PGE_2 without IL-1α. Lane 4, treated with 100 μM of forskolin without IL-1α. Lane 5, stimulated with 100 U/ml of IL-1α. Lane 6, treated with 1 μg/ml of PGE_1 in combination with 100 U/ml of IL-1α. Lane 7, treated with 1 μg/ml of PGE_2 in combination with 100 U/ml of IL-1α. Lane 8, treated with 100 μM of forskolin in combination with 100 U/ml of IL-1α. Lane 9, molecular weight marker.

DISCUSSION

NO is generously considered to affect the cartilage metabolism in pathological condition. The immunohistochemical examination showed that both iNOS and COX-2 were expressed repletely in the cartilage of RA patients, while they were scarcely expressed in health cartilage. There is a possibility that NO and PGs might have influence on each other in inflammatory process. For instance, NO induces COX-2 and hence NO increases PG production in mouse macrophage cell line RAW264.7.[6] Whether either NO

or PG is an upper mediator, varies from cell to cell. However, it is still unclear in terms of the articular chondrocyte. Then we examined the effects of PGE_2 on NO synthesis in articular chondrocytes.

Nitric oxide release of articular chondrocytes induced by IL-1α was partially inhibited by cyclooxygenase-2 inhibitor. Exogenously added PGE_2 caused synergistic enhancement of IL-1α induced NO synthesis. In bovine articular chondrocytes as well as iNOS mRNA, we have also detected COX-2 mRNA expression by IL-1α (data not shown). We have, therefore, hypothesized that PGE_2 generated by IL-1α-induced COX-2 might elevate intracellular cAMP level in articular chondrocyte and this elevation of cAMP might upregulate iNOS mRNA expression by either autocrine or paracrine mechanism. Therefore, we examined the effect of direct activator of adenylate cyclase, forskolin on NO generation. Forskolin has augmented NO release from the articular chondrocytes in the presence of IL-1α in the similar manner to PGE_2.

These data here, however, do not ensure the elevation of cAMP can directly augment iNOS gene expression by IL-1α. Either changes in the transcription or in the mRNA stability may be accounted for the changes in mRNA levels. In vascular smooth muscle cell, the stimulation of transcription of iNOS gene by cAMP appears to be the most likely mechanism involved and enhanced expression of iNOS by elevating cAMP concentration is not attributed to the increased mRNA stability on the basis of experiments using actinomycin D to stop transcription.[15] Cyclic-AMP is known to regulate the expression of a number of genes, and many of them have been shown to possess common DNA elements, the cAMP response element or activator protein 2 element, which are usually present in the 5'-upstream region of these genes.[24] Since iNOS has consensus sequences of phosphorylation site for protein kinase A (PKA),[21] it is still possible that an increase in NO production by cAMP-elevating agents is partly mediated by phosphorylation of iNOS by PKA.

Protein kinase C (PKC) system is also considerable as other intracellular mediator of iNOS gene expression. Phorbol-12-myristate-13-acetate (PMA), an activator of PKC had not significant effect on NO production and H7, an inhibitor of PKC failed to suppress chondrocyte NO release by IL-1α (data not shown). Activation of NF-κB by tyrosine kinase seems to play an important role in iNOS mRNA induction of murine liver cell line, BNL CL.2[19] or murine macrophage by IL-1.[28] Mitogen activated protein kinase (MAP kinase) is reported to be another significant mediator of iNOS gene expression in rat mesangial cell.[10]

Though further investigation should be required for detecting the detail intracellular mechanism of iNOS gene expression of articular chondrocyte, these data stated here also prove that non-steroidal anti-inflammatory drugs including COX-2 inhibitor might have therapeutic effects on the inflammatory disease such as RA or OA by inhibiting not only PGs synthesis but also NO production.

In summary, prostaglandins might modulate the articular cartilage metabolism by augmentation of chondrocyte NO synthesis in inflammatory process through cAMP system as its amplifier.

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