行政院國家科學委員會專題研究計畫　成果報告

分析細胞激素、藥物試劑及玻尿酸，對骨性關節炎之半月板、軟骨以及滑膜組織培養中基質金屬蛋白水解酶

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-040-013-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：中山醫學大學醫學系

計畫主持人：呂克修
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報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 11 月 2 日

Regulation of Gelatinases Expression by Cytokines, Endotoxin and Pharmacological Agents in the Human Osteoarthritic Knee

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Running title: Regulation of Gelatinase in OA Knee

Abstract

We examined the amount of gelatinases (matrix metalloproteinase-2 and -9, MMP-2 and -9) in chondral, meniscal and synovial cultures of early osteoarthritis (OA) after treatment with or without catabolic cytokines (interleukin-1α, IL-1α and tumor necrosis factor-α, TNF-α), lipopolysaccharide (LPS), and pharmacological agents (plasmin/serine proteinase antagonist aprotinin, protein synthesis inhibitor cycloheximide, and protein kinase C (PKC) inhibitors staurosporine, H7 and Gö6976) for investigation of the effects on MMP-2 and -9 production in OA. Gelatin zymography revealed that IL-α, TNF-α and LPS could elevate MMP-2 secretion in all tissue cultures and also increase MMP-9 production in all synovial and some meniscal cultures. In contrast, aprotinin, cycloheximide, staurosporine, H7 and Gö6976 could suppress MMP-2 secretion in all tissue cultures and also decrease MMP-9 production in all synovial and some meniscal cultures. Our data indicate that catabolic cytokines and LPS increase the effect of tissue destruction and disintegration of extracellular matrix in early OA. Agents that target the PKC pathway, plasmin/serine proteinase or protein synthesis for MMP-2 and -9 in early OA inhibit MMPs production. These findings might contribute to therapeutic efficacy.

Keywords: MMP-2; MMP-9; osteoarthritis; cytokine; endotoxin; pharmacological agent

INTRODUCTION

For cartilage metabolism there are two pathways which are ascribable to enzymic digestion of the extracellular matrix (ECM). Firstly, an intrinsic pathway by which chondrocytes themselves degrade cartilage ECM and, secondly, an extrinsic pathway by which tissues or cells other than chondrocytes, such as inflamed synovium, pannus tissues, and infiltrated inflammatory cells, break down the ECM of cartilage mostly through synovial fluid. Osteoarthritis (OA) is characterized by degenerative changes due to a gradual loss of cartilage ECM that may result from inappropriate mechanical loads[1], elevated levels of proinflammatory cytokines[2], mutational changes affecting cartilage components[3], and increased levels of matrix proteolysis[4].

Local release of catabolic cytokines from synovial membrane, particularly interleukin-1 (IL-1)[5,6] and tumor necrosis factor-α (TNF-α)[7], in response to tissue damage and inflammation can markedly alter the metabolic activity of chondrocytes and inhibit glycosaminoglycan synthesis. In OA, levels of these cytokines are known to be elevated. IL-1 has been found to contribute to the disease process by stimulating the biosynthesis of proteolytic enzymes[8] and increasing matrix metalloproteinases (MMPs) mRNA expression[9], and also by inhibiting the production of ECM constituents such as type II collagen and proteoglycans from articular cartilage[10,11]. Therefore, inhibition of

IL-1 and its actions by IL-1 receptor antagonist via gene therapy and antibodies against IL-1 are beneficial in reducing the symptoms of arthritis including invasion of cartilage by synovium[12-14]. Blocking IL-1 induced MMP-3 and MMP-13 enzymes or their gene expression by novel physiologic and pharmacological inhibitors is also an important therapeutic approach for arthritis[15]. Some of these effects of IL-1 are mediated by MMPs.

The MMPs are a family of zinc- and calcium dependent proteases which function in the degradation and remodeling of ECM proteins during different developmental processes such as organ morphogenesis, bone formation, angiogenesis, and remodeling during reproductive processes, as well as in pathological processes such as inflammation, chronic degenerative diseases and tumor invasion[16]. They comprise at least five main categories, including gelatinases (MMP-2 and -9), collagenases (MMP-1, -8, and -13), stromelysins (MMP-3, -10, and -11), membrane-type (MT) MMPs (MMP-14, -15, -16, and -17), and others (MMP-7 and -12). Gelatinases A and B (MMP-2 and -9) play a critical role in inflammatory infiltration and degradation of the basement membrane, a structure that largely composes of type IV collagen and separates the epithelial and stromal compartments. They are secreted as latent precursor enzymes and can be activated by limited proteolysis, which results in a loss of molecular weight of about 10 kDa. Different signaling cascades are involved in MMP

regulation, depending on the stimulus, cell type, and the MMP. All three mitogen-activated protein (MAP) kinase signaling pathways have been demonstrated to be involved in human MMP-2 and -9 gene regulation. Although, several laboratories have investigated that induction of MMP-2 and -9 syntheses in various cells involves multiple signaling cascades, including extracellular signal-regulated kinase (ERK), p38 kinase, c-Jun NH_2-terminal kinase (JNK), proteinase kinase C (PKC), and phospholipase D (PLD) signaling pathways[17-21], little is known about the regulatory mechanisms of MMP-2 and -9 in OA.

Thus far, the increased expressions of MMP-2 and -9 in the synovium of patient with arthritic effusions superiorly reflected the inflammatory condition of the joints[22-24]. A positive correlation between the production of MMP-9 and a rapid destruction of the hip joint with OA was found[25]. The zymographic profile and MMP expression can be a useful diagnostic adjunct in patients with OA, providing precise information on the condition of the articular cartilage and the breakdown of its matrix[26,27]. Previously, we have also investigated that MMP-2, -9 and MT1-MMP (MMP-14), significantly increased mRNA levels in lesional areas of early osteoarthritic chondral, meniscal and synovial tissues than that in paralesional areas, play a role in physiologic and pathologic events[28]. To date, the mechanisms of MMP-2 and -9 expressions *in vivo* in OA have not been fully elucidated. A better understanding of these signaling and regulatory

mechanisms may lead to novel strategies for suppressing the MMP-2 and -9 expressions via blocking the signal transduction pathways. The aim of the study was to examine the effects of catabolic cytokines (IL-1α and TNF-α), lipopolysaccharide (LPS), and pharmacological agents (plasmin/serine proteinase antagonist aprotinin, protein synthesis inhibitor cycloheximide, and PKC inhibitors staurosporine, H7 and Gö6976) on MMP-2 and -9 production in early osteoarthritic chondral, meniscal and synovial cultures using gelatin zymography.

MATERIALS AND METHODS

Chemicals and Reagents

IL-1α, TNF-α, LPS, aprotinin, cycloheximide, staurosporine, H7 and Gö6976 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All culture materials were obtained from Gibco (Grand Island, NY, USA). IL-1α, TNF-α, LPS, aprotinin, cycloheximide and Gö6976 were directly dissolved in the culture medium, whereas H7 was first dissolved in 100% ethanol and then diluted with the culture medium. Stock solutions of staurosporine were prepared in dimethyl sulfoxide and stored at -20℃. The final concentration of each solvent in the medium did not exceed 0.25% (v/v). At these concentrations, the solvents used were not toxic to human osteoarthritic chondral, meniscal and synovial cultures. The final concentrations of IL-1α, TNF-α, LPS,

aprotinin, cycloheximide, staurosporine, H7 and Gö6976 used in this study were 10 ng/ml, 10 ng/ml, 1μg/ml, 100 μg/ml, 10 μM, 5 μM, 10 μM and 2 μM, respectively.

Sampling and Chondral, Meniscal and Synovial Cultures

Using the Arthroforce® III forceps (Karl Storz, Germany), more than 500 mg specimens of cartilage, menisci and synovia were obtained from the patients with primary early OA knees undergoing arthroscopic débridement at our hospital by the same author[28]. All patients gave informed consent for their surgical specimens to be studied. All of the specimens were divided and weighted equally 50 mg, then transferred into 24 tissue culture flasks respectively and incubated at 37℃ under a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% penicillin-streptomycin (10,000 U/ml) and 10 mg/ml streptomycin as described by Hsieh *et al*[28].

Cytokine, Endotoxin and Pharmacological Agent Treatments

The chondral, meniscal and synovial tissues were cultured for 3 h, at which time the medium was changed to a medium treated with or without appropriate concentrations of catabolic cytokines (IL-1α and TNF-α), LPS, and pharmacological agents (plasmin/serine proteinase antagonist aprotinin, protein synthesis inhibitor

cycloheximide, and PKC inhibitors staurosporine, H7 and Gö6976). The conditioned medium samples were collected on 3 h, 24 h, 48 h and 96 h, and prepared for gelatin zymography as described by Hsieh *et al*[28].

Gelatin Zymography

Gelatin zymography was performed according to a protocol developed by Kleiner and Stetler-Stevenson[29]. Of each effusion sample or culture medium, 20 μl of effusions or media containing 10 μg of total protein was loaded onto a precast sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin. After electrophoresis, gels were processed as described by Chou *et al*[30]. Nonstaining bands representing the activities of latent and activated forms of MMP-2 and -9 were quantitatively measured by spot density measurement using a digital imaging analysis system (Alpha Innotech, Mt. Prospect, IL). Results were calculated as integrated density value (IDV). IDV is the sum of all the pixel values after background correction, i.e., IDV = Σ (each pixel value – background value) as described by Lu *et al*[23] and Hsieh *et al*[28]. Then the levels of MMP-2 and -9 from treated groups were expressed as optical density (% of control) in comparison with the control group. More than three replicates of each concentration were performed in each test. All assays were repeated three times to ensure reproducibility.

Statistical Analysis

All statistical calculations were performed using Student's *t*-test for control and treated groups. Statistical significance was set at $p < 0.05$.

RESULTS

Figure 1 is the zymograms of conditioned medium samples from human osteoarthritic chondral, meniscal and synovial cultures co-treated with or without catabolic cytokines (IL-1α and TNF-α), LPS, and pharmacological agents (plasmin/serine proteinase antagonist aprotinin, protein synthesis inhibitor cycloheximide, and PKC inhibitors staurosporine, H7 and Gö6976) on 24 h. The gelatin zymograms revealed that the proMMP-2 migrated at 72 kDa in all chondral, meniscal and synovial cultures. ProMMP-9 was also observed at 92 kDa regions in all synovial and some meniscal cultures. The activated form of MMP-2 and -9 showed a loss of the propeptide of about 10 kDa respectively.

Effect of Drugs on MMP-2 Level

The latent and activated forms of MMP-2 presented in all chondral, meniscal and

synovial cultures. Generally, the levels of them in IL-1α and TNF-α treated medium samples on 3 h seemed to have few effects in all chondral, meniscal and synovial cultures. (Table I-III) They significantly increased on day 1, and these elevated levels persisted through to day 4. The stimulation of LPS in all chondral, meniscal and synovial cultures also showed the increased MMP-2 production in a time-dependent manner. In contrast, aprotinin, cycloheximide, and PKC inhibitors staurosporine, H7 and Gö6976 could decrease the secretion of latent and activated forms of MMP-2 in all chondral, meniscal and synovial cultures. Their effects depended on the duration of exposure.

Effect of Drugs on MMP-9 Level

ProMMP-9 presented in all synovial and some meniscal cultures. In IL-1α and TNF-α treated groups, the levels of proMMP-9 significantly increased in a time-dependent manner in synovial cultures since day 1, but not significantly in meniscal cultures. (Table II and III) Similarly, this observation was found in LPS treated medium samples. In contrast, in aprotinin, cycloheximide, and PKC inhibitors staurosporine, H7 and Gö6976 treated groups, the levels of proMMP-9 significantly decreased in a time-dependent manner in synovial cultures since day 1, but not significantly in meniscal cultures. The levels of activated MMP-9 that only appeared in synovial

cultures since day 1 consistently elevated in IL-1α, TNF-α and LPS treated groups and suppressed in aprotinin, cycloheximide, staurosporine, H7 and Gö6976 treated groups since day 2. (Table III) Again, the effects were all in a time-dependent manner. Actually, the activated MMP-9 could be detected as a small white zone in all zymographies of undiluted effusions in all synovial cultures on 3 h.

DISCUSSION

Human articular cartilage is continually remodeled both during growth and development and during the whole life. OA has complex etiology and causes degenerative changes of articular cartilage or subchondral bone and loss of function. Furthermore, there is strong circumstantial evidence that MMP-2 participates in the turnover of normal cartilage matrix, whereas MMP-9 and some MMP-2 facilitate the progressive destruction of the cartilage matrix in OA[28]. The resulting imbalance between the anabolic and catabolic activities leads to the severe degradation of articular cartilage observed in OA[6].

Chondrocytes are targets of IL-1α and TNF-α, which inhibit their anabolic activities (ECM synthesis) and trigger catabolic pathways[31] while concomitantly upregulating MMP gene expression[32]. In this short-term *ex vivo* model, which provides

a system representative of the *in vivo* environment, the MMP up-regulation of proinflammatory cytokines in osteoarthritic cartilage, menisci and synovial tissues may occur in *in vivo*. These results support the concept of cytokine-MMP associations[33,34]. However, the mechanisms of signal transduction are still not completely understood and need to be further investigated.

LPS or endotoxin, which is the major component of the outer surface of Gram-negative bacteria and a potent activator of cells (macrophages/monocytes, endothelial cells, types A and B synoviocytes) of the inflammatory response[35], is the most potent natural inducer of MMP biosynthesis and secretion by various cell type. In monocytes and macrophages, LPS stimulate both MMP-2 and -9 at the transcriptional and protein levels. Similarly, LPS up-regulated MMP-2 and -9 expressions in all tissue cultures of this study. Since the most recent studies suggest that LPS signaling cascade uses an analogous molecular framework for signaling as IL-1[36], LPS induced MMP-2 and -9 expressions via an IL-1 pathway may exist.

Plasmin has been shown to activate MMP-2 *in vitro*[37]. Additionally, plasmin is a participant in the proteolytic cascade resulting in activation of stromelysins and, in this way, in activation of MMP-9[38]. In order to determine whether the regulatory mechanisms also involved in OA, we examined the ability of aprotinin, a plasmin/serine proteinase antagonist, to affect MMP-2 and -9 secretion. It is interesting to note that

aprotinin significantly decreased the production and secretion of MMP-2 and -9 in human osteoarthritic tissue cultures. Moreover, the amount of MMP-2 and -9 were suppressed by cycloheximide, a protein synthesis inhibitor, indicating the participation of translation processes and the necessity of protein synthesis. These results clearly show that plasmin and the *de novo* synthesis of some proteinaceous mediators are required for the regulation of MMP-2 and -9 in OA. Treatment with the proteinase antagonist or protein synthesis inhibitor may decrease MMP-2 and -9 to attack the ECM whether it is normal or not.

The messenger-signaling pathways that effect MMP gene expression are still incompletely understood. The PKC signaling pathway is known to be important in MMP expression and secretion[19,39.40]. To determine whether PKC was involved in the production of MMP-2 and -9 in OA, osteoarthritic chondral, meniscal and synovial tissues were incubated with or without PKC inhibitors staurosporine, H7 and Gö6976. We found that they consistently down-regulated MMP-2 and -9 secretion into the medium from all tissue cultures. Gö6976, which is selective for isozyme subtypes α, β, and μ, is highly specific for PKC, whereas nonselective PKC inhibitors staurosporine and H7 have less specific for PKC, inhibiting also serine/threonine protein kinases[41-42]. This fact implies that, under the conditions in these studies, the regulation of MMP-2 and -9 may be mediated via a PKC signaling pathway in OA knee.

However, other pathways, such as three major MAP kinase signaling pathways (ERK, p38 kinase and JNK) and PLD signaling pathway, remain unclear and should be fully elucidated.

Unlike other published studies, ours investigates patients with early OA knees undergoing arthroscopic débridement. Our data indicate that proinflammatory cytokines and LPS up-regulate MMP-2 and -9 secretion and increase the effect of tissue destruction and disintegration of ECM in early OA. Thus, activation of MMPs may be one of the distinct host degradative pathways in the pathogenesis of early OA. In contrast, pharmacological agents that target the PKC pathway, plasmin/serine proteinase or protein synthesis for MMP-2 and -9 in chondral, meniscal and synovial tissues of early OA inhibit MMPs production, and such inhibition may contribute to the pathomechanism of OA. These findings may be useful for developing possible new therapeutic attempts, involving the use of drugs that modulate host-response mechanisms to inhibit or suppress MMP-mediated tissue destruction in OA. Further detailed studies, however, should be undertaken to clarify the agents that can also regulate other MMPs, such as MMP-3 and MMP-13, produced or secreted by human osteoarthritic tissues.

Acknowledgments

This work was supported by grant from the Research Section of Chung Shan Medical University (CSMU 92-OM-B-017) and National Science Council, Taiwan (NSC92-2314-B-040-013).

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Figure Legends

FIGURE 1

Gelatinase levels in osteoarthritic chondral (A), meniscal (B) and synovial (C) cultures co-treated with or without catabolic cytokines (IL-1α and TNF-α), LPS, and pharmacological agents (plasmin/serine proteinase antagonist aprotinin, protein synthesis inhibitor cycloheximide, and PKC inhibitors staurosporine, H7 and Gö6976) on 24 h were quantified by gelatin zymography.

FIGURE 1

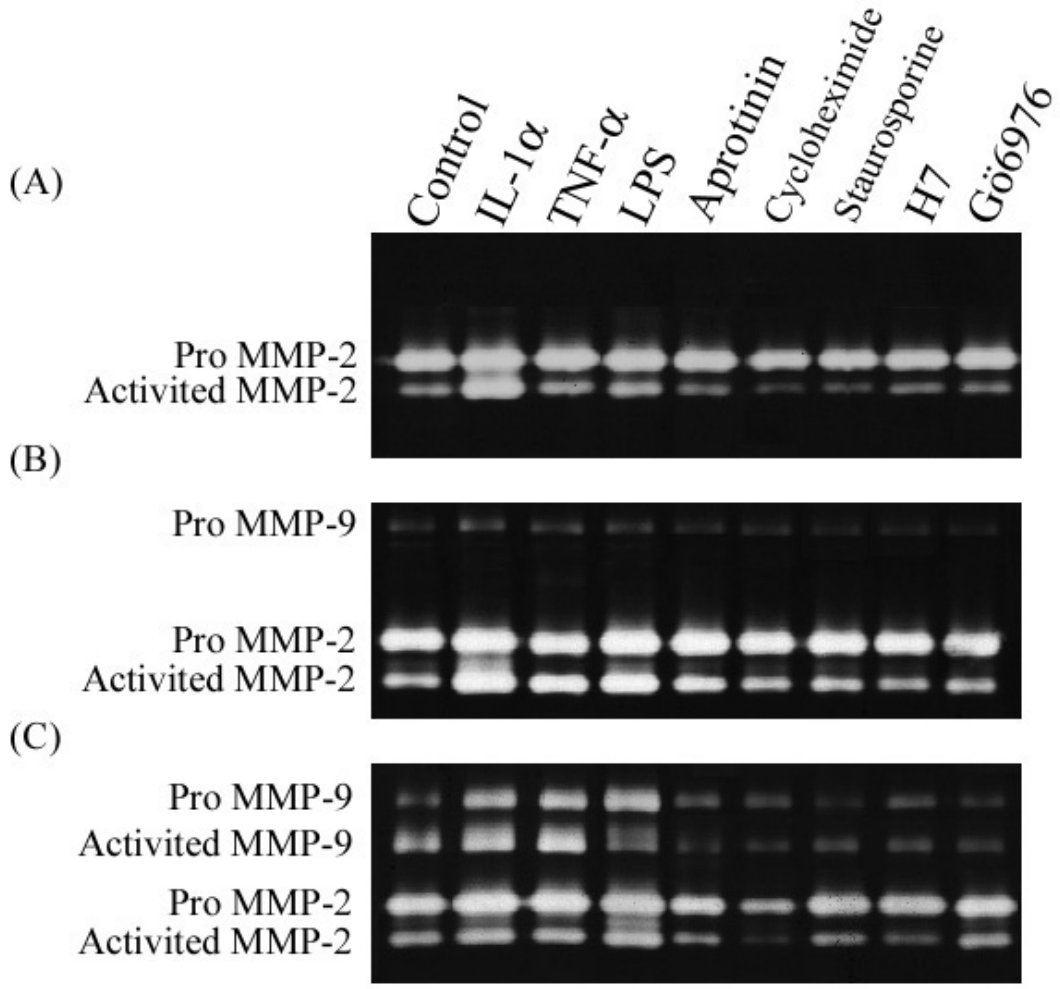
Control
IL-1α
TNF-α
LPS
Aprotinin
Cycloheximide
Staurosporine
H7
Gö6976
(A)
Pro MMP-2
Activited MMP-2
(B)
Pro MMP-9
Pro MMP-2
Activited MMP-2
(C)
Pro MMP-9
Activited MMP-9
Pro MMP-2
Activited MMP-2

TABLE I Levels of MMP-2 in chondral cultures after treatment with or without catabolic cytokines (IL-1α and TNF-α), LPS, and pharmacological agents (plasmin/serine proteinase antagonist aprotinin, protein synthesis inhibitor cycloheximide, and PKC inhibitors staurosporine, H7 and Gö6976)

	ProMMP-2 (% of control)	Activated MMP-2 (% of control)
IL-1α		
3 h	129.20 ± 7.13**	149.05 ± 25.81*
24 h	172.50 ± 25.91*	227.45 ± 55.37*
48 h	230.93 ± 26.41**	241.75 ± 38.19**
96 h	312.93 ± 42.02**	353.95 ± 58.42**
TNF-α		
3 h	110.48 ± 11.69	106.15 ± 5.50
24 h	126.88 ± 16.07*	113.60 ± 16.66
48 h	158.25 ± 14.34**	150.70 ± 23.86*
96 h	205.23 ± 24.22**	187.80 ± 14.31***
LPS		
3 h	112.03 ± 9.79	123.68 ± 9.29*
24 h	198.90 ± 23.46**	148.00 ± 16.28**
48 h	269.13 ± 22.02***	273.78 ± 60.07**
96 h	284.03 ± 54.58**	346.30 ± 111.88*
aprotinin		
3 h	88.00 ± 7.84	95.08 ± 3.81
24 h	85.28 ± 11.50	91.58 ± 6.89
48 h	83.03 ± 10.73	69.68 ± 15.70*
96 h	77.05 ± 8.60*	62.30 ± 12.62**
cycloheximide		
3 h	86.63 ± 7.50*	84.73 ± 7.91*
24 h	73.65 ± 5.19**	64.95 ± 4.90***
48 h	51.03 ± 8.25***	38.83 ± 8.09***
96 h	47.75 ± 16.92**	36.75 ± 5.49***
staurosporine		
3 h	89.55 ± 6.40*	96.48 ± 5.45
24 h	80.98 ± 9.30*	71.50 ± 3.63***
48 h	79.68 ± 10.37*	64.90 ± 11.18**
96 h	78.35 ± 5.18**	53.93 ± 7.92***
H7		
3 h	93.00 ± 6.99	89.58 ± 2.91**
24 h	85.33 ± 8.71*	69.43 ± 11.01*
48 h	71.25 ± 7.15**	71.78 ± 6.47**
96 h	67.88 ± 5.92**	60.38 ± 9.77**
Gö6976		
3 h	93.68 ± 5.61	88.75 ± 3.86**
24 h	91.45 ± 10.42	70.05 ± 18.63*
48 h	87.30 ± 11.01	77.58 ± 10.65*
96 h	90.83 ± 3.21*	66.93 ± 15.08*

Values are mean ± S.D. (n = 4-6). Statistical significance different from control values (control = 100%): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

TABLE II Levels of MMP-2 and -9 in meniscal cultures after treatment with or without catabolic cytokines (IL-1α and TNF-α), LPS, and pharmacological agents (plasmin/serine proteinase antagonist aprotinin, protein synthesis inhibitor cycloheximide, and PKC inhibitors staurosporine, H7 and Gö6976)

	ProMMP-2 (% of control)	Activated MMP-2 (% of control)	ProMMP-9 (% of control)
IL-1α			
3 h	137.85 ± 34.23	263.03 ± 83.66*	175.95 ± 14.92
24 h	209.58 ± 60.17*	294.85 ± 88.55*	320.55 ± 47.59
48 h	312.20 ± 50.23**	313.45 ± 86.99*	322.35 ± 28.50
96 h	482.68 ± 89.65**	361.50 ± 91.25*	541.03 ± 169.63*
TNF-α			
3 h	130.23 ± 26.13	133.78 ± 18.09*	117.20 ± 15.13
24 h	188.10 ± 14.38***	197.30 ± 8.54***	177.35 ± 12.80
48 h	187.73 ± 9.09***	198.55 ± 21.44**	211.40 ± 7.07*
96 h	222.60 ± 50.11*	287.83 ± 40.00**	253.87 ± 102.69
LPS			
3 h	113.15 ± 13.49	114.93 ± 18.81	183.75 ± 21.00
24 h	130.55 ± 10.48**	162.70 ± 22.62*	188.15 ± 16.48
48 h	178.80 ± 7.27***	168.48 ± 29.93*	245.50 ± 31.11
96 h	189.98 ± 16.18**	193.45 ± 32.14**	227.80 ± 58.13
aprotinin			
3 h	93.50 ± 5.33	90.75 ± 5.78*	103.05 ± 9.26
24 h	86.80 ± 4.81*	79.70 ± 2.89***	92.90 ± 4.95
48 h	80.58 ± 6.10**	75.63 ± 4.31***	88.25 ± 1.34
96 h	73.65 ± 6.56**	70.53 ± 10.10**	87.17 ± 9.74
cycloheximide			
3 h	90.70 ± 6.13	88.80 ± 5.32*	93.60 ± 5.66
24 h	83.03 ± 3.38**	76.78 ± 4.30**	92.55 ± 5.44
48 h	81.55 ± 4.35**	65.78 ± 6.81**	82.60 ± 4.24
96 h	75.43 ± 6.16**	77.58 ± 1.85***	63.63 ± 16.45
staurosporine			
3 h	97.78 ± 8.11	91.30 ± 7.40	101.40 ± 6.65
24 h	86.78 ± 9.82	88.85 ± 7.91	94.05 ± 6.43
48 h	71.33 ± 4.52***	79.45 ± 9.96*	73.50 ± 7.07
96 h	62.98 ± 6.53***	74.05 ± 4.25***	84.00 ± 6.97
H7			
3 h	97.55 ± 3.79	92.28 ± 8.06	100.65 ± 3.32
24 h	90.28 ± 5.52*	87.85 ± 4.53*	92.55 ± 8.56
48 h	82.70 ± 5.23**	76.10 ± 4.47**	92.90 ± 4.81
96 h	81.13 ± 4.84**	68.98 ± 10.41**	83.97 ± 4.65*
Gö6976			
3 h	96.98 ± 5.82	87.50 ± 3.47**	98.10 ± 1.84
24 h	89.13 ± 5.78*	83.10 ± 4.79**	91.50 ± 8.63
48 h	79.85 ± 6.60**	77.60 ± 10.88*	84.10 ± 7.78
96 h	67.45 ± 9.78**	64.33 ± 10.80**	85.43 ± 11.07

Values are mean ± S.D. (n = 4-6). Statistical significance different from control values (control = 100%): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

TABLE III Levels of MMP-2 and -9 in synovial cultures after treatment with or without catabolic cytokines (IL-1α and TNF-α), LPS, and pharmacological agents (plasmin/serine proteinase antagonist aprotinin, protein synthesis inhibitor cycloheximide, and PKC inhibitors staurosporine, H7 and Gö6976)

	ProMMP-2 (% of control)	Activated MMP-2 (% of control)	ProMMP-9 (% of control)	Activated MMP-9 (% of control)
IL-1α				
3 h	138.08 ± 6.51***	128.07 ± 32.46	151.00 ± 20.51	N.D.
24 h	172.55 ± 16.96**	198.18 ± 61.69*	176.53 ± 17.56*	175.00 ± 16.26
48 h	247.08 ± 41.96**	326.15 ± 123.74*	278.73 ± 37.93**	268.65 ± 48.51**
96 h	334.45 ± 57.15**	335.50 ± 45.39**	351.65 ± 61.77**	306.75 ± 47.69**
TNF-α				
3 h	103.05 ± 18.09	118.37 ± 15.98	109.95 ± 8.98	N.D.
24 h	160.40 ± 37.11*	171.03 ± 25.65*	132.30 ± 10.42*	132.00 ± 4.81
48 h	219.70 ± 50.61*	259.83 ± 76.68*	195.95 ± 23.65**	311.33 ± 66.12**
96 h	329.75 ± 47.62**	293.15 ± 44.17**	320.25 ± 53.67**	362.55 ± 83.36**
LPS				
3 h	117.43 ± 10.63*	123.93 ± 8.40*	132.30 ± 5.52	N.D.
24 h	222.28 ± 31.88**	168.83 ± 32.40*	149.77 ± 18.55*	153.15 ± 15.06
48 h	333.75 ± 71.62**	260.80 ± 43.51**	211.38 ± 23.55**	173.45 ± 34.07*
96 h	340.95 ± 29.94***	278.93 ± 50.73**	314.68 ± 19.73***	250.63 ± 32.36**
aprotinin				
3 h	94.28 ± 7.46	89.87 ± 5.75	93.05 ± 4.88	N.D.
24 h	82.80 ± 6.18*	76.93 ± 11.59*	84.00 ± 4.68*	77.15 ± 13.22
48 h	79.50 ± 8.61*	57.23 ± 3.68***	81.38 ± 7.41*	80.95 ± 9.31*
96 h	60.03 ± 11.79**	55.10 ± 6.97***	69.80 ± 5.56**	68.60 ± 4.48***
cycloheximide				
3 h	88.25 ± 2.86**	85.17 ± 5.06*	84.60 ± 7.07	N.D.
24 h	61.83 ± 12.61**	67.93 ± 7.42**	67.87 ± 8.01*	70.20 ± 8.91
48 h	50.55 ± 10.07**	58.60 ± 7.69**	40.65 ± 5.71***	64.65 ± 6.61**
96 h	42.10 ± 12.23**	31.50 ± 11.28***	31.80 ± 11.44***	51.08 ± 10.15**
staurosporine				
3 h	98.63 ± 7.00	93.23 ± 6.60	91.05 ± 2.05	N.D.
24 h	85.48 ± 14.44	77.53 ± 7.76**	83.03 ± 5.66*	81.70 ± 7.35
48 h	76.83 ± 7.42**	64.13 ± 5.71***	73.18 ± 10.19*	68.90 ± 10.92*
96 h	77.03 ± 7.50**	56.38 ± 14.22**	60.33 ± 7.16**	59.95 ± 4.03***
H7				
3 h	98.68 ± 2.65	94.00 ± 4.50	94.40 ± 2.69	N.D.
24 h	87.90 ± 9.35	90.73 ± 4.57*	86.70 ± 2.72*	86.85 ± 0.50*
48 h	81.28 ± 9.48*	83.18 ± 4.84**	75.08 ± 8.75*	75.93 ± 7.74**
96 h	81.18 ± 8.79*	69.05 ± 4.55***	62.63 ± 9.57**	77.95 ± 8.54*
Gö6976				
3 h	92.23 ± 6.22	94.37 ± 3.87	97.45 ± 1.63	N.D.
24 h	80.60 ± 5.49**	87.68 ± 1.62***	69.40 ± 6.17*	90.05 ± 3.46
48 h	77.68 ± 4.91**	75.45 ± 5.46**	59.68 ± 10.33**	76.98 ± 8.80*
96 h	77.43 ± 6.63**	57.98 ± 17.43*	65.05 ± 6.12***	71.65 ± 6.28**

Values are mean ± S.D. (n = 4-6). Statistical significance different from control values (control = 100%): *p < 0.05, **p < 0.01, ***p < 0.001.

N.D. = not detectable (see “Results”).