Induction of Stromelysin Gene Expression by Tumor Necrosis Factor α Is Inhibited by Dexamethasone, Salicylate, and N-Acetylcysteine in Synovial Fibroblasts¹

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ABSTRACT
Prolinflammatory cytokines, altered connective tissue metabolism, and overexpression of matrix metalloproteinases (MMPs) such as stromelysin compared to tissue inhibitors of metalloproteinases (TIMPs) result in synovial inflammation and erosion of arthritic cartilage. Tumor necrosis factor α (TNF-α) is a major synovial inflammatory mediator responsible for inhibiting extracellular matrix (ECM) synthesis and stimulating degradation of cartilage ECM by activated MMPs in arthritic joints. To suppress these effects and to gain insight into the mechanism of TNF-α action, we identified the inhibitors of TNF-α stimulation of stromelysin gene expression. In bovine synovial fibroblasts, TNF-α did not affect a recently identified inhibitor, TIMP-3, but induced stromelysin mRNA expression in a dose- and time-dependent fashion (3- to 5-fold) which required de novo protein synthesis. Stimulation by TNF-α was potently inhibited (99–100%) by the synthetic glucocorticoid, dexamethasone. Sodium salicylate dose-dependently inhibited (100%) the TNF-α action. Indomethacin and ibuprofen were partially inhibitory. Free radical scavenger antioxidant, N-acetylcysteine (but not other antioxidants) also suppressed the TNF-α induction (36–100%) of stromelysin suggesting involvement of reactive oxygen species in the induction process. TNF-α induction of stromelysin gene expression can therefore be inhibited at the gene expression level by several pharmacological agents which are likely to function via arachidonic acid metabolites, free radical scavenging or interference with the activator protein 1, polyoma virus enhancer A-binding protein 3, and nuclear factor κB classes of transcription factors. Our results may help to elucidate the mechanism of TNF-α action and explain the beneficial role of these agents in the treatment of inflammatory diseases.

Tumor necrosis factor α (TNF-α) is the major inflammatory mediator in arthritis that invokes multiple responses and regulates numerous genes implicated in inflammation (Vilcek and Lee, 1991). It is found at high levels in the synovial fluids of patients with arthritis, whose synovium exhibits different degrees of inflammation (Westacott and Sharif, 1996). TNF-α inhibits transcription of cartilage-specific type-II, -IX and -XI collagens in human chondrocytes and contributes to cartilage loss in joint diseases (Regnato et al., 1993). Osteoarthritic (OA) cartilage is more susceptible to stimulation by TNF-α due to increased p55 TNF-α receptor on the chondrocytes (Westacott and Sharif, 1996). TNF-α inhibits embryonal chondrocytic phenotype and turns them into fibroblast-like cells in vitro (Mohamed-Ali, 1995). Overall, TNF-α promotes tissue destruction by metalloenzymes and prevents tissue repair.

Matrix metalloproteinases (MMPs) consist of collagenases, stromelysins, gelatinases, and membrane-type MMPs, which can degrade extracellular matrix during its physiological (e.g., animal development) and pathological (arthritis, cancer, cardiovascular diseases) turnover. Activity of MMPs is regulated by the tissue inhibitor of metalloproteinase (TIMP) gene family which consists of four members named TIMP-1, -2, -3, and -4 (Birkedal-Hansen, 1995). MMPs not only cause erosion of arthritic joints but also process TNF-α into its mature secreted active form (Gearing et al., 1994). Inhibitors of MMPs prevent this processing. Stromelysin is a major MMP, responsible for degradation of cartilage aggrecan and activation of procollagenase (Birkedal-Hansen, 1995). The expression of this gene is increased in arthritic joints (Zafarullah et al., 1993) and in unstable atherosclerotic plaques.

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ABBREVIATIONS: AP-1, activator protein-1; FCS, fetal calf serum; PEA-3, polyoma virus enhancer A-binding protein 3; MMP, matrix metalloproteinase; NAC, N-acetylcysteine; NMA, N-monomethyl-L-arginine; PDTC, pyrrolidine dithiocarbamate; TIMP, tissue inhibitors of metalloproteinase; TNF-α, tumor necrosis factor α.
(Galis et al., 1994). Since synovial inflammation is the major
discomfort in arthritis, and the synovial fibroblasts are the
main target cell-type, this study was aimed at identifying
inhibitors of stromelysin upregulation by TNF-α in the
bovine synovial fibroblast model in order to understand the
mechanism of TNF-α action. We tested the hypothesis that
the inhibitors of protein synthesis, transcription factors, and
reactive oxygen species may block TNF-α stimulation of
stromelysin gene expression.

Materials and Methods

Reagents. Dulbecco’s modified Eagle’s medium (DMEM), fetal
calf serum (FCS), antibiotic-antimycotic agents, and agarose were
obtained from Canadian Life Technologies Inc. (Gibco-BRL, Burling-
ton, Ontario, Canada). Plasticware such as 100-mm plates and T-75
flasks were purchased from Nunc, Inc. (Roskilde, Denmark). Tumor
necrosis factor α was obtained from R&D systems (Minneapolis, MN)
and Boehringer Mannheim (Laval, Quebec). Cycloheximide was ob-
tained from U.S. Biochemical Corporation (Cleveland, OH). Collage-
nase type II, dexamethasone, sodium salicylate, N-acetylcysteine,
indomethacin, ibuprofen, N-methyl-L-arginine, α-tocopherol, ascor-
bic acid, and pyridilidone dithiocarbamate were purchased from
Sigma Chemical Company (St. Louis, MO). Digoxigenin (DIG) RNA
labeling and chemiluminescence systems were obtained from Boe-
ringer Mannheim. Hybrid nylon membrane was purchased from
Amersham Canada. RNA probe labeling kits were obtained from
Promega (Madison, WI). Restriction endonucleases were purchased
from Pharmacia Biotech. Inc. (Baie d’urfe, Quebec, Canada). Other
common laboratory reagents were purchased from Fisher Scientific
(Montreal, Canada).

Synovial Fibroblast Culture. Normal bovine synovial mem-
branes were dissected from the freshly slaughtered adult animals
from a local abattoir. The tissues were washed extensively in large
volumes of 0.9% NaCl containing 5× antibiotic-antimycotic solution
and further washed five times with PBS containing 5× penicillin-
streptomycin and 1× fungizone (Gibco-BRL, Burlington, Ontario,
Canada). Synovial fibroblasts were released by dissociation with
trypsin and collagenase (Sigma type II) for 6 h in DMEM at 37°C.
The cells were collected by centrifugation and washed three times
with PBS and plated as primary monolayer cultures until adherence
and confluent growth were attained. These cells were passaged sev-
eral times to obtain a homogeneous population of synovial fibro-
blasts. Before different treatments, cells were kept in 0.5 or 5% serum
containing DMEM for 24 h and then exposed to different reagents in
the same medium for various periods of time described in the text.

RNA Extraction and Northern Blot Hybridization Analysis.
Total RNA was extracted by a rapid procedure (Chomczynski
and Sacchi, 1987) and aliquots of 5 μg were analyzed by electrophoretic
fractionation in 1.2% formaldehyde-agarose gels. The integrity and
quantity of applied RNA were verified by ethidium bromide staining
of the gels and photography of the 28S and 18S ribosomal RNA
bands. The RNA was electrophoretically transferred onto nylon membrane
using a Bio-Rad Transblot in the presence of 1× standard saline
citrate buffer at a current of 500 mAmp for 12 h. Complete transfer
was ascertained by ethidium bromide staining of the gel. Northern
blots were hybridized as described previously (Zafarullah et al.,
1993) with a human stromelysin cDNA probe generously provided by
Dr. Richard Breathnach (Muller et al., 1988). This probe which
hybridizes with bovine stromelysin mRNA, was a 1.6-kbp EcoRI-
EcoRI cDNA fragment cloned in the plasmid pGEM-4Z (Promega
Biotec, Madison, WI). The vector was linearized with NruI and RNA
probe synthesized with T7 polymerase according to the protocols of
Promega Biotec. The bovine TIMP-3 probe (Su et al., 1996) was a
2.042-kbp EcoRI-EcoRI cDNA fragment cloned in the plasmid
pGEM-4Z (Promega Biotec). The vector was linearized with SacI and
the RNA probe was synthesized with SP6 polymerase according to
the protocols of Promega Biotec. The glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) cDNA probe (from American Type Culture
Collection, Rockville, MD) described earlier (Zafarullah et al.,
1993). The human 28S ribosomal RNA plasmid (American Type
Culture Collection) was digested with XbaI and a probe was synthe-
size using T7 polymerase. The probes were labeled with the digoxi-
genin (DIG) RNA labeling kit containing DIG-11-UTP followed by
prehybridization, hybridization, and detection of Northern blots with
the DIG Luminescent Detection kit of Boehringer Mannheim accord-
ing to their protocols. Alternatively the probes were labeled to high-
specific activity (1 × 10⁶ cpm/μg) with [α-32P]CTP (3000 Ci/mmol;
DuPont-NE (Boston, MA). Densitometric values from three exper-
iments (obtained with Alpha Imager, Canberra-Packard, Meriden,
CT) of stromelysin bands were divided by those of GAPDH bands to
give an average quantitative estimate of inhibition as depicted by bar
graphs.

Results

Before testing the potential inhibitors, we investigated
whether the normal bovine synovial fibroblasts were respon-
sive to TNF-α. To that end, these cells were exposed for 24 h
to different concentrations (50–1000 U/ml) of TNF-α in me-
dium containing either minimal 0.5% or 5% FCS. The RNA
was hybridized with a human stromelysin probe which cross-
hybridizes with the bovine stromelysin mRNA of the same
size. Although the lower doses were effective, following nor-
malization with the constitutive GAPDH or 28S control RNA,
stromelysin mRNA was 3- to 4-fold inducible by TNF-α at 200
U/ml and 150 U/ml in 0.5% FCS and 4- to 5-fold inducible in
5% FCS (Fig. 1). The RNA levels of a housekeeping
GAPDH gene (Fig. 1, lower panel) and those of another control gene,
28S rRNA (not shown), were not changed by the treatments.
Therefore, TNF-α is a potent inducer of stromelysin gene
expression in bovine synovial fibroblasts.

To determine the time course of stromelysin mRNA expres-
sion, cells were treated with TNF-α (150 U/ml) for different
time periods under 0.5% FCS (Fig. 2A) or 5% FCS (Fig. 2B).
Cells were also treated for 16 h at a higher dose (1000 U/ml)
as a positive control. As shown in Fig. 2A, the maximal
(3.4-fold) induction of stromelysin mRNA expression oc-
curred between 16 and 24 h. The pattern of induction was
similar under 5% FCS conditions (2.5–3.75-fold), although
overall levels of RNA were some what lower than those under
0.5% conditions. To investigate the inducibility of a recently

![Fig. 1. Stromelysin mRNA expression in response to different doses of
TNF-α. Lanes 0, DMEM containing 0.5% or 5% FCS-treated bovine
synovial fibroblasts. Other lanes represent cells treated in the same
medium with different concentrations of TNF-α (U) for 24 h. The
GAPDH RNA serving as loading and constitutively expressed control is
shown at the bottom.](https://i.imgur.com/3.png)
characterized metalloproteinase inhibitor, TIMP-3, by TNF-α. Cells were kept in DMEM containing 0.5 (A) or 5% (B) FCS (lanes 0) or exposed to indicated doses of TNF-α for different time periods and RNA analyzed with DIG-labeled stromelysin and TIMP-3 probes and detected by chemiluminescence systems. The positions of the stromelysin (upper panels) and the three TIMP-3 transcripts (in kb) (middle panels) are indicated. The GAPDH RNA bands serving as loading and constitutively expressed control are shown at the bottom.

Fig. 2. Time-dependent increase in stromelysin mRNA by treatment of bovine synovial fibroblasts with TNF-α. Cells were kept in DMEM containing 0.5% or 5% FCS (lanes 0) or exposed to indicated doses of TNF-α for different time periods and RNA analyzed with DIG-labeled stromelysin and TIMP-3 probes and detected by chemiluminescence systems. The positions of the stromelysin (upper panels) and the three TIMP-3 transcripts (in kb) (middle panels) are indicated. The GAPDH RNA bands serving as loading and constitutively expressed control are shown at the bottom.

Fig. 3. Effect of cycloheximide on the induction of stromelysin mRNA by TNF-α. Lane control, untreated cells; lane 2, TNF-α (150 U/ml); lane 3, cycloheximide (CHX, 10 μg/ml); lane 4, cycloheximide and TNF-α. All treatments were for 24 h. GAPDH RNA bands serving as a loading and unaffected control are shown in the middle panel. The densitometric scanning data from three experiments are shown as stromelysin (strom.)/GAPDH ratio at the bottom. The levels of stromelysin induction were treated as 100% and the average inhibition is shown as bar graphs.

with a synthetic glucocorticoid, dexamethasone (1–2 μg/ml). In three separate experiments, this agent potently suppressed (99–100%) stromelysin induction by TNF-α, reducing its mRNA to basal level (Fig. 4). GAPDH RNA levels were not inhibited.

Aspirin-like agents or salicylates are commonly used drugs to treat inflammation in arthritis. They can induce heat shock transcription factor and inhibit TNF-α-induced nuclear factor κB (NF-κB) activation (see Discussion). We examined whether this drug influenced the induction of stromelysin gene by TNF-α. Cells were treated with sodium salicylate (2–20 mM) alone or in combination with TNF-α. It dose-dependently down-regulated TNF-α induction of stromelysin gene expression in three separate experiments (Fig. 5, upper panel). Following correction with the consistent GAPDH control RNAs, 100% inhibition was observed (Fig. 5, lower panel).

Since glucocorticoids and salicylates can influence prostaglandin metabolism, we further examined the latter’s involvement by exposing cells to other cyclooxygenase inhibitors, indomethacin and ibuprofen alone or with TNF-α. Indomethacin and ibuprofen partially inhibited (48–50%) stromelysin RNA induction by TNF-α in a reproducible fashion (Fig. 6). Since nitric oxide (NO) activates stromelysin, and salicylates can inhibit inducible nitric oxide synthase (iNOS), we examined whether iNOS inhibitor could reduce TNF-α-
induced stromelysin induction. N-methyl-l-arginine (NMA), an inhibitor of iNOS did not decrease but further increased this induction (1 mM). Thus, arachidonic acid metabolites (but not NO) appear to partially mediate this induction in our system.

To investigate the role of reactive oxygen species in TNF-α induction of stromelysin gene, synovial fibroblasts were treated either with TNF-α or with a thiolic antioxidant, N-acetylcysteine (NAC) alone or in combination. GAPDH and 28S RNA (not shown) levels were unaffected by the treatments. In three experiments, this agent, at concentrations of 20 to 30 mM, strongly down-regulated stromelysin mRNA induction by TNF-α in a dose-dependent fashion as depicted in the representative Fig. 7 (upper panel). An inhibition of 36% (at 10 mM) to 100% (at 20–30 mM) was observed with NAC (Fig. 7, lower panel). Other antioxidants such as α-tocopherol, ascorbate, and pyrrolidine dithiocarbamate (PDTC) did not block TNF-α induction of stromelysin. PDTC by itself induced stromelysin mRNA and actually further enhanced induction by TNF-α (Fig. 8).

**Discussion**

In this report, we demonstrated that the proinflammatory cytokine, TNF-α, up-regulates stromelysin gene without affecting TIMP-3 gene expression in mammalian synovial fibroblasts. The induction process requires de novo protein synthesis and may involve activation of transcription factors. We further showed that the induction can be inhibited or down-regulated by dexamethasone, an aspirin-like drug and an antioxidant, possibly via inhibition of arachidonic acid metabolites, antioxidant activity, and interference with the activator protein 1 (AP-1) and NF-κB classes of transcription factors.

The demonstrated stromelysin gene induction by TNF-α in bovine synovial fibroblasts is in agreement with other cell types where stromelysin was inducible by this factor. In bovine nasal chondrocytes, TNF-α induced both proteinases and prostaglandin E2 (Smith et al., 1992). This factor transcriptionally induced collagenase and stromelysin expression in porcine chondrocytes by mediation of staurosporine- and H7-sensitive protein kinases distinct from protein kinase C.
The two major cell types in joints are clearly the targets of TNF-α action. A lack of TIMP-3 mRNA induction by TNF-α observed here is in contrast with a previous report where TNF-α induced TIMP-3 expression in mouse cells in the presence of 5% FCS serum (Sun et al., 1995). In our cells, TIMP-3 mRNA could not be markedly induced in the presence of 5% or 0.5% serum with different brands of TNF-α. The lack of TIMP-3 induction by TNF-α is in accord with another study were TIMP-1 (another family member) was not induced but was suppressed by this agent in synovial cells (Shingu et al., 1993). MMP induction and TIMPs inhibition (or noninduction) by TNF-α can contribute to enzyme-inhibitor imbalance commonly observed in arthritic tissues (Dean et al., 1989).

Suppression of TNF-α induction of stromelysin by cycloheximide is similar to that of collagenase inhibition by this agent in human fibroblasts (Sciacolino et al., 1994). Therefore, up-regulation is through indirect mechanisms. The prolonged induction of the c-jun protooncogene by TNF-α was proposed as one of the intermediate transcription factors that is part of the AP-1 complex comprising of c-fos/c-jun factors. This occurs by upstream prolonged activation of c-jun kinase which phosphorylates the transactivation domain of c-Jun (Westwick et al., 1994). The promoter DNA-binding activities of AP-1 proteins are increased by TNF-α (Sciavolino et al., 1994). AP-1 binding sites are found in the human collagenase and stromelysin 1 promoters (Buttice et al., 1991). Contrary to a previous report, we did not observe induction of stromelysin by cycloheximide alone (Otani et al., 1990) in bovine cells.

The inhibition of TNF-α action by the anti-inflammatory glucocorticoid in synovial fibroblasts is particularly relevant to arthritis as synovial inflammation is a major discomfort for the patients, and glucocorticoids are used to relieve the symptoms in patients and in animal models of arthritis. These agents stop further erosion of cartilage in vivo by metalloproteinases (Pelletier et al., 1994). Inhibition of collagenase and stromelysin mRNA by dex was also observed in human diploid FS-4 fibroblasts (Sciavolino et al., 1994). We showed that TIMP-3 gene induction by transforming growth factor β is also inhibited by dexamethasone (Su et al., 1996). The molecular mechanism of inhibition possibly involves direct interaction between c-jun and glucocorticoid receptor.
Inhibition of TNF-Induced Stromelysin Expression

Effect of other antioxidants on stromelysin mRNA induction by TNF-α. Synovial fibroblasts were pretreated with the indicated doses of α-tocopherol, ascorbic acid, and PDTC individually for 30 min or followed by additional TNF-α treatment for 24 h. Following hybridizations with stromelysin and GAPDH probes (upper and middle panels), the average densitometric values from three experiments were plotted as bar graphs (bottom panel).

proteins resulting in prevention of c-jun binding to the AP-1 sequences in stromelysin promoter (Yang-Yen et al., 1990). Another recently proposed mechanism of immunosuppressive and anti-inflammatory action of glucocorticoids is the induction of IkBα gene product which associates with TNF-α-activated NF-κB (dissociated NF-κB from IkBα) factor, thus preventing its translocation to the nucleus (Scheinman et al., 1995). The applicability of this model for stromelysin inhibition is possible, since NF-κB and its translocation from cytoplasm to the nucleus is inhibited by high doses of sodium salicylate and aspirin (Kopp and Ghosh, 1994) which some how influences stromelysin gene expression. Activated NF-κB is commonly found in joints of OA and RA patients (Marok et al., 1996). AP-1 and polyoma virus enhancer A-binding protein 3- (PEA-3) (but not NF-κB) binding sites have been detected in the known stromelysin promoters. An additional possibility is that at higher concentrations, salicylate acted by its antioxidant type of activity (Dinis et al., 1994) and scavenged TNF-α-induced hydroxyl radicals. This ability alone could be sufficient to inhibit stromelysin induction by TNF-α.

Aspirin is an old wonder drug that reduces the risk of heart attacks and colon cancer (Thun et al., 1991). Aspirin and sodium salicylate have neuroprotective abilities against glutamate toxicity by inhibiting NF-κB activation (Grilli et al., 1996). Stromelysin is involved in arthritic cartilage erosion, instability of atherosclerotic plaques (Galas et al., 1994), and metastatic invasion of cancer cells. Our demonstration of inhibition of stromelysin gene expression by aspirin-like drugs suggests a possible beneficial effect of this drug in these disorders and may explain the results of epidemiological studies on aspirin. Inhibition of TNF-α action is a major target for suppressing inflammation. In experimentally induced arthritis in rabbits, neutralization of TNF-α with its antibody suppressed inflammatory but not the cartilage resorptive aspect of arthritis, suggesting its key role in inflammation (Lewithtwaite et al., 1995). Our study, utilizing stromelysin as a target, has demonstrated alternative inhibitors of TNF-α action.

Inhibition of stromelysin mRNA induction by a reactive oxygen species scavenger NAC suggests that reactive oxygen species are involved in stromelysin induction by TNF-α. Oxidant by-products are used by phagocytes to combat infections during immune response, but are also a source of oxidative damage in degenerative diseases of aging (Ames et al., 1993). Indeed, a lower serum antioxidant level has been recognized as a risk factor for rheumatoid arthritis (Heliövaara et al., 1994). Stromelysin mRNA inhibition by NAC may be a result of upstream inhibition of redox-sensitive c-fos and c-jun transcription factor expression by this antioxidant (Lo et al., 1996). These AP-1 factors along with PEA-3 binding factors are important regulators of the transcription of human stromelysin promoter (Buttice et al., 1991). Other antioxidants such as α-tocopherol, ascorbate, and PDTC were drugs which are commonly prescribed for the management of arthritis. Aspirin-like drugs inhibit COX-2 induction by TNF-α and constitutive COX-1 (Geng et al., 1995), which through some unknown mechanism may inhibit stromelysin gene expression observed here. Other nonsteroidal anti-inflammatory drugs such as Naproxen and Tenidap also inhibit collagenase mRNA expression in a canine model of arthritis (Fernandes et al., 1995). Our results suggest that prostaglandins are partly involved in TNF-α induction of stromelysin. Alternatively, TNF-α could induce nitric oxide synthase (NOS) responsible for the synthesis of NO which could activate collagenase and stromelysin (Murrell et al., 1995). Aspirin-like drugs at pharmacological (1–3 mM) and suprapharmacological (IC₅₀ = 20 mM) concentrations can inhibit inducible NOS (Amin et al., 1995). Treatment with NOS inhibitor, NMA, however, failed to block TNF-α action in our system. A third possibility is that TNF-α-induced activation of NF-κB and its translocation from cytoplasm to the nucleus is inhibited by high doses of sodium salicylate and aspirin (Kopp and Ghosh, 1994) which some how influences stromelysin gene expression. Activated NF-κB is commonly found in joints of OA and RA patients (Marok et al., 1996). AP-1 and polyoma virus enhancer A-binding protein 3- (PEA-3) (but not NF-κB) binding sites have been detected in the known stromelysin promoters. An additional possibility is that at higher concentrations, salicylate acted by its antioxidant type of activity (Dinis et al., 1994) and scavenged TNF-α-induced hydroxyl radicals. This ability alone could be sufficient to inhibit stromelysin induction by TNF-α.

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not inhibitory, but rather stimulatory (particularly PDTC), suggesting distinct mechanisms of action for different antioxidants. This may be due to the reported ability of PDTC to induce AP-1 transcription factors whose targets are found in the stromelysin promotor (Yokoo and Kitamura, 1996). Interestingly, induction of cartilage catabolism by fibroenectin fragments by MMPs is also inhibited by antioxidants (Homandberg et al., 1996). NAC also blocks nuclear translocation of NF-κB in rheumatoid synovial fibroblasts (Sakurada et al., 1996).

In summary, we have demonstrated that stromelysin gene expression is upregulated by TNF-α. Inhibition of this augmentation by cycloheximide, glucocorticoids, salicylates, and NAC suggests that the induction is mediated by de novo protein synthesis and intermediate steps such as prostaglandins, reactive oxygen species, and AP-1, PEA-3, or NF-κB transcription factor activation. These results also give a better insight into the mechanism of action of these drugs, some of which are beneficial in the treatment of arthritis, cardiovascular diseases, and cancer.

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References


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