PHARMACOLOGICAL INFLUENCE ON THE BIOSYNTHESIS AND ACTIVITY OF PLASMIN, PLASMINOGEN ACTIVATORS AND THEIR INHIBITOR PAI-1

**INTRODUCTION**
Production and activation of proteases, such as matrix metalloproteinases (MMPs), the recently identified aggrecanases (ADAMTS4 and ADAMTS11), and the plasminogen activator (PA)/plasmin system are involved in the cartilage breakdown process during chronic inflammatory arthritides and osteoarthritis. The PAs are serine proteases, which catalyse the activation of plasminogen to plasmin. Two isoforms of PA exist: tissue-type PA (tPA) and urokinase-type PA (uPA). MMPs are synthesized and released from chondrocytes as inactive proenzymes, which are activated by limited proteolytic cleavage. Plasmin is a possible candidate involved in the activation process of these enzymes. Plasmin further contributes to cartilage matrix degradation by its direct proteolytic activity on extracellular macromolecules, such as proteoglycans. The PA activity is controlled by 2 distinct plasminogen activator inhibitors (PAIs), PAI-1 and PAI-2. Since one therapeutic approach in blocking the proteolytic destruction of articular cartilage can be the inhibition of enzymes responsible for the activation of MMPs, our presented study focus on the ability of drugs, used in the treatment of arthritic disorders, to inhibit the biosynthesis and/or activity of PAs and plasmin. As net proteolytic activity depends also on the amount of specific inhibitors, such as PAI-1, we also investigated the effects of these drugs on the biosynthesis of this inhibitor.

**METHODS**

1. **In-vitro-activity of PAs and plasmin:** At a concentration of 10^(-8) M (or lower) 7 drugs of the following pharmacological groups were tested for their ability to inhibit the activity of purified human plasmin, uPA and tPA: (1) nonsteroidal anti-inflammatory drugs (NSAIDs), and (2) glucocorticoids. The activity of uPA and tPA was determined by the rate of plasmin generated from plasminogen. Two isoforms of PA exist: tissue-type PA (tPA) and urokinase-type PA (uPA). MMPs are synthesized and released from chondrocytes as inactive proenzymes, which are activated by limited proteolytic cleavage. Plasmin is a possible candidate involved in the activation process of these enzymes. Plasmin further contributes to cartilage matrix degradation by its direct proteolytic activity on extracellular macromolecules, such as proteoglycans. The PA activity is controlled by 2 distinct plasminogen activator inhibitors (PAIs), PAI-1 and PAI-2. Since one therapeutic approach in blocking the proteolytic destruction of articular cartilage can be the inhibition of enzymes responsible for the activation of MMPs, our presented study focus on the ability of drugs, used in the treatment of arthritic disorders, to inhibit the biosynthesis and/or activity of PAs and plasmin. As net proteolytic activity depends also on the amount of specific inhibitors, such as PAI-1, we also investigated the effects of these drugs on the biosynthesis of this inhibitor.

2. **Biosynthesis of uPA, tPA and PAI-1:**
   a) **Cell culture:** Chondrocytes from the metacarpophalangeal joints of 18-24 month old steers were isolated by enzymatic digestion and encapsulated in alginate beads. Beads were suspended in supplemented Ham’s F12 medium with 10% FBS. Cultures were maintained for 8 days at 37°C, 5% CO2 and 95% humidity. Serum was slowly reduced until day 6. During the final 48 hours cells were fed with a serum free media supplemented with the substrate and, in addition, H-D-Val-Leu-Arg-pNA by plasmin (Verheijen et al., 1982, 1988). The activity of plasmin was measured using 3H-radiolabeled proteoglycan monomers as substrate and, in addition, H-D-Val-Leu-Arg-pNA.
   b) **The activity of PAs within cell culture:** The PA activity was determined by the method of Verheijen (1982, 1988). In a coupled photometric assay, the conversion of plasminogen to plasmin is recorded by the hydrolysis of a chromogenic substrate (S-2251), which is specific for plasmin. Enzyme activity was calculated by generation of a standard curve according to a method described by Verheijen (1982, 1988). In a coupled photometric assay, the conversion of plasminogen to plasmin is recorded by the hydrolysis of a chromogenic substrate (S-2251), which is specific for plasmin. Enzyme activity was calculated by generation of a standard curve according to a method described by Verheijen (1982, 1988).
   c) **Quantitation of mRNA expression:** Total RNA was isolated and reverse transcribed into a first strand cDNA. Amplification primers, that are specific for the cDNAs of the investigated proteins, were used. PCR was performed in the presence of digoxigenin(DIG)-11-dUTP. DIG-labeled PCR products were analyzed with a PCR-ELISA.
   d) **Quantitation of PAI-protein:** PAI-1 protein was determined with an ELISA. The detection limit was 0.5 ng/ml PAI-1.

3. **Statistical analysis:** The whole experiments were repeated 6-12 times. Groups of data were analyzed using student’s one-tailed paired t-test. Significance was set p<0.05. Data are presented as mean ± SD.

**RESULTS**

1. **Effects of IL-1 alpha:** Untreated chondrocytes released significant levels of PA activity which was increased 1.3-fold (p<0.05) by IL-1. Evaluation of the mRNA expression of the two isoforms revealed, that uPA was enhanced 1.8-fold (p<0.01), whereas the transcript level of tPA was induced 3.2-fold (p<0.01) by this cytokine. No effects could be observed on the protein content of PAI-1, although mRNA transcription of this inhibitor was significantly increased (2.6-fold, p<0.01).

2. **Effects of dexamethasone and NSAIDs on the activity of PAs and plasmin as well as on the expression of PAs and PAI-1:** All NSAIDs (acetylsalicylic acid, diclofenac-Na, indomethacin, meloxicam, naproxen, tiaprofenic acid) and the glucocorticoid tested showed the potential to strongly inhibit the activity of PAs within the cell culture model. In addition, all tested NSAIDs and dexamethasone dose-dependently inhibited the mRNA expression of tPA, whereas only indomethacin, tiaprofenic acid and the glucocorticoid were also able to reduce the expression of uPA. Furthermore, dexamethasone, indomethacin, meloxicam, naproxen, and tiaprofenic acid enhanced the protein level of PAI-1. None of the drugs tested were able to inhibit in vitro the activity of purified human plasmin and of the PAs.

**DISCUSSION**
In our study, the expression of both isoforms of PAs as well as of PAI-1 was significantly enhanced by IL-1 indicating that this cytokine might not alter the ratio between the levels of PAs and their inhibitor PAI-1.

The therapeutic relevance of the differential inhibitory effects of NSAIDs on the expression of PAs must be considered with respect to the different roles in cartilage metabolism that have been described for tPA and uPA. For instance, some agreement exists that uPA is the predominant protein product of PAI-1, whereas the presence of fibrin in synovium and cartilage is one of the pathologic features of rheumatoid arthritis as it may impede normal nutrition of these tissues. In our experiments acetylsalicylic acid, naproxen and tiaprofenic acid inhibited PA activity at concentrations which are attainable in vivo. Their restricted effectiveness towards tPA expression, however, could result in protraction of fibrin removal rather than in inhibition of uPA mediated degradation of articular cartilage. In contrast, dexamethasone inhibited the expression of both isoforms, which might further contribute to its antidegenerative effects, as it could lead to a reduced activation of latent MMPs.

Dexamethasone and meloxicam dose-dependently increased immunoreactive PAI-1 and this effect was pretranslational as confirmed by RT-PCR. Upon treatment with indomethacin, naproxen and tiaprofenic acid only the protein content of this inhibitor was enhanced, whereas the mRNA level remained unchanged. Thus, we assume that their effect might have derived from a suppression of uPA-receptor expression leading to a reduced clearance of PAI-1 from the culture media. Compared to the drug levels found in vivo, the stimulation of PAI-1 by dexamethasone, meloxicam, naproxen and tiaprofenic acid may occur in vivo. Meloxicam has recently proven to be effective against cartilage degradation in an animal model of rheumatoid arthritis. According to our data, reduction of PA activity by increasing PAI-1 might be a possible mechanism by which this antidegenerative effect is achieved.

In conclusion, our study clearly demonstrate that NSAIDs and the glucocorticoid dexamethasone act not only against joint disease symptoms like pain and inflammation, but can also interfere with processes that underlie the destruction of articular cartilage during osteoarthritis and rheumatoid arthritis. Furthermore, the marked differences of the tested NSAIDs with respect to their ability to modulate the imbalance between proteases and inhibitors suggests, that the respective modes of action are independent of the inhibition of cyclooxygenases.

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