Minocycline Inhibits the Production of Inducible Nitric Oxide Synthase in Articular Chondrocytes

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ABSTRACT. ***Objective.*** To determine the *in vitro* effects of tetracyclines and nonsteroidal antiiflammatory drugs on interleukin 1α (IL-1α) induced NO production and biosynthesis of inducible NO synthase (iNOS) by articular chondrocytes.

Methods. Bovine chondrocytes were cultured in alginate beads. Cells were treated with IL-1α in the presence or absence of drugs at various concentrations. Expression of mRNA for iNOS was analyzed by reverse transcription polymerase chain reaction-ELISA. Protein synthesis of iNOS was determined by immunoprecipitation. NO production was taken as a measure for the activity of the enzyme.

Results. Minocycline dose dependently reduced IL-1 stimulated NO production by inhibition of the mRNA expression (IC_{50} = 69.9 µM) and protein synthesis (IC_{50} = 37.11 µM) of iNOS. Diclofenac-Na at a concentration of 10 µM only weakly reduced nitrite accumulation and mRNA expression of iNOS. No effects were observed for tetracycline, doxycycline, and meloxicam.

Conclusion. Inhibition of iNOS in articular chondrocytes may be a new mechanism by which minocycline could exert its beneficial effects in the treatment of joint diseases. (J Rheumatol 2001;28:336–40)

Key Indexing Terms:
TETRACYCLINES NONSTEROIDAL ANTIINFLAMMATORY DRUGS
NITRIC OXIDE INDUCIBLE NITRIC OXIDE SYNTHASE ARTICULAR CHONDROCYTES

Destruction of articular cartilage is a common feature in joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). The structural breakdown of proteoglycans and collagen is believed to be the result of an imbalance in the anabolic and catabolic activities of chondrocytes. Recent observations have proposed nitric oxide (NO) as an important mediator responsible for these changes in chondrocyte metabolism. For instance, elevated levels of NO have been found in the synovial fluid, cartilage, and serum of patients with arthritis[1]. Excess production of NO has been associated with decreased synthesis of aggrecan[2] and collagen type II[3]. Further, NO enhanced matrix metalloproteinase (MMP) activity *in vitro*[4], and was shown to reduce the synthesis of interleukin 1 (IL-1) receptor antagonist in chondrocytes[5]. Expression of the inducible form of NO synthase (iNOS) was detected in synoviocytes and chondrocytes from patients with inflammatory arthritides[6]. This notion is supported by the observation that cultured chondrocytes from different species synthesize large amounts of NO following exposure to IL-1 and a limited number of other stimuli[7,8].

The identification of NO as a major catabolic factor in joint diseases prompted studies on the therapeutic usefulness of inhibition of iNOS activity. Thus the synthetic iNOS inhibitor N^G-methyl-L-arginine suppressed the development of arthritis when administered prophylactically in a rat model of adjuvant induced arthritis[9]. In 2 recent studies, Pelletier, *et al*[10,11] have shown in an experimental OA dog model that the selective inhibition of iNOS reduces the progression of cartilage lesions, as well as the production of MMP and IL-1. Results from these animal studies indicate that identification of agents that could reduce the activity of iNOS might form the basis for potential new therapies in the treatment of OA and RA. So far, however, only a few studies have dealt with pharmacological modulation of NO production in chondrocytes. Attur, *et al*[12] found that some nonsteroidal antiinflammatory drugs (NSAID) reduced the accumulation of NO in the media of bovine chondrocytes stimulated with cytokines. On the other hand, NO production by human chondrocytes was partly reduced by dexamethasone and methylprednisolone[13].

Clinical studies have revealed that tetracylines are effective in the treatment of RA[14]. Several possible mechanisms have been proposed, including inhibition of MMP activity and expression[15], angiogenesis[16], and immunomodulatory effects on human mononuclear cells[17]. We examined whether tetracylines might be effective against the IL-1 induced NO production in articular chondrocytes. As inhibi-

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tion of iNOS has emerged as a new mode of action of NSAID[18], we also evaluated effects of diclofenac-Na and meloxicam on iNOS expression and NO production.

MATERIALS AND METHODS

Materials. Pronase® and protein G PLUS/protein A agarose were purchased from Calbiochem (Bad Soden, Germany), ascorbate, penicillin, streptomycin, L-glutamine, alpha-ketoglutarate, phenylmethylsulfonyl fluoride (PMSF), and mouse IgG were obtained from Sigma (Deisenhofen, Germany). Low range protein molecular weight marker, DIG labeling mix, and polymerase chain reaction (PCR) ELISA kits were from Boehringer (Mannheim, Germany), alginate from Kelco (London, UK), and collagenase CLS-2 was purchased from Worthington (Freehold, Lakewood, NJ, USA). Ham's F12, fetal bovine serum (FBS), gentamycin, and Trizol® were obtained from Life Technologies (Eggenstein, Germany), human recombinant IL-1α from R&D Systems (Wiesbaden, Germany). M-MLV reverse transcriptase and Taq polymerase were purchased from Promega (Mannheim, Germany), CR-ITS+™ was from Collaborative Research (Bedford, UK), and Easy Taq™ express protein labeling mix [^{35}S] from NEN (Dreieich, Germany). Mouse anti-iNOS antibody was obtained from Transduction Laboratories (Lexington, UK). Drugs were generous gifts from pharmaceutical companies: Tetracycline-HCl (Lederle GmbH & Co.), doxycycline (Pfizer GmbH), minocyline (Azupharma GmbH), diclofenac-Na (Ciba Geigy AG), meloxicam (Boehringer Ingelheim).

Culture of chondrocytes. Chondrocytes were isolated from macroscopically healthy metacarpophalangeal joints of 18–24 month old steers and subsequently encapsulated in alginate beads. Cells were released from the matrix by initial digestion with pronase (0.8% w/v) for 1 h at 37°C followed by collagenase (0.5% w/v) digestion for 4 h at 37°C. Viability of the harvested cells as assessed by trypan blue exclusion test was always > 95%. Chondrocytes were suspended in sterile alginate solution (1.2% w/v) to a final concentration of 3×10^6 cells/ml alginate. The preparation of alginate beads was as described[19]. Twenty beads per well were transferred to a 12 well culture dish containing 2.5 ml/well growth medium (Ham's F12 containing 10% FBS, 25 mM HEPES, 0.3 mg/ml L-glutamine, 0.03 mg/ml alpha-ketoglutarate, 0.05 mg/ml ascorbic acid, 10 U/ml penicillin, and 0.1 mg/ml streptomycin). Media were changed every second day. Cultures were maintained for 8 days at 37°C, 5% CO_2, 95% humidity. Serum was slowly reduced until Day 6. During the final 48 h culture medium containing the serum substitute CR-ITS$^+$ instead of FBS and IL-1α at a final concentration of 0.5 ng/ml was given to the cells either alone or together with tetracyclines (1 µM to 100 µM in H_2O), NSAID (10 µM), or the drug vehicle, respectively. After the incubation period media were collected and stored frozen at –20°C. Cells were harvested by solubilization of the alginate beads in citrate buffer (55 mM sodium citrate and 150 mM NaCl, pH 7.2, 1 ml per 20 beads) with subsequent centrifugation. The alginate supernatant was stored frozen at –20°C until analyzed.

Determination of NO production. Nitrite levels in media and solubilized alginate samples were measured using the Griess reaction with sodium nitrite as standard. Briefly, culture samples were mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 25% H_3PO_4) and incubated 10 min at room temperature. Optical density was measured at 523 nm in an ELISA photometer.

Determination of iNOS and total protein synthesis. iNOS protein synthesis was analyzed by metabolic labeling of chondrocytes followed by immunoprecipitation. Cells were radiolabeled with 50 µCi/ml [^{35}S]methionine/cysteine during the final 18 h of the incubation period with IL-1, drugs, or drug vehicle. Alginate beads were solubilized in citrate buffer and the released chondrocytes were resuspended in lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 2 mM PMSF, pH 7.4). After centrifugation the protein containing supernatant was precleared by incubation with nonimmune mouse IgG and protein G PLUS/protein A agarose suspension for 1 h. Mouse anti-iNOS antibody (1 µg/ml) was added to the samples. After overnight incubation at 4°C, immune complexes were precipitated by the addition of a protein G PLUS/protein A agarose suspension. Precipitates were washed 3 times in Tris buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.4), boiled for 5 min in Laemmli buffer, and then separated on 8% sodium dodecyl sulfate-polyacrylamide gels under reducing conditions. Gels were stained with Coomassie brilliant blue R250 for visualization of molecular weight marker proteins, fixed, and dried. The radioactivity of the single protein band at 116 kDa was measured with the Automatic TLC-Linear Analyzer LB284/LB285 (Berthold, Wildbad, Germany). Peaks were analyzed with Chroma 1D software (Berthold).

For determination of synthesis of total protein, cells were lysed as described above. After centrifugation, proteins in the supernatant were precipitated by the addition of 0.5 M perchloroacetic acid (PCA). Precipitates were applied to Whatman GF/C filters and extensively washed with 0.25 M PCA. Filters were dried and the filter associated radioactivity was quantified by liquid scintillation counting.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from chondrocytes using Trizol according to the manufacturer's instructions. For generation of single stranded cDNA 0.25 µg RNA was incubated with 50 pmol oligo-dT primer and 200 units M-MLV reverse transcriptase for 90 min at 42°C with a final denaturation step at 95°C for 15 min. The cDNA was diluted in sterile water and then stored at –20°C. Primer pairs for iNOS and GAPDH were chosen from the published sequences[20,21] and used for amplification: iNOS: 5′-TAGAGGAACATCTGGCCAGG-3′, 5′-TGGCAGGGTCCCCTCTGATG-3′, GAPDH: 5′-GAGATGGACCCTTTTGG-3′, 5′-GTGAAGGTCGGAGTCAACG-3′.

Depending on the cDNA to be amplified, PCR was performed for 22 cycles (GAPDH) and 24 cycles (iNOS), respectively (denaturing at 95°C for 45 s, annealing for 60 s, extension at 72°C for 120 s). The reaction was thereby kept in the exponential range of amplification to obtain semiquantitative results (data not shown). PCR products were labeled with digoxigenin (DIG) by addition of a DIG labeling mix to the reaction mixture. PCR products were commercially sequenced (MWG Biotech, Ebersberg, Germany), which revealed their identity to the predicted sequences (see above).

PCR-ELISA. DIG labeled PCR products were quantitated with a PCR ELISA kit according to the manufacturer's instructions. Briefly, DIG labeled PCR products were immobilized to streptavidin coated microtiter plate wells by hybridization to a biotinylated oligonucleotide that was complementary to a ~20 bp spanning sequence in the inner part of the DIG labeled PCR product. The bound hybrids were detected by an anti-DIG peroxidase conjugate and by use of the colorimetric substrate ABTS®. Absorbance was read in an ELISA photometer at 405 nm and was taken as a measure for the amount of amplified product. Expression of GAPDH was taken as internal control.

Statistical analysis. All experiments were repeated 4–12 times. Groups of data were analyzed using Student's one-tailed paired t test. Significance was set at $p \leq 0.05$. Data are presented as mean ± SD.

RESULTS

Effect of IL-1α on biosynthesis and activity of iNOS in articular chondrocytes. Initially, chondrocytes were treated with various concentrations of IL-1α (data not shown). The concentration of cytokine (0.5 ng/ml) used in this study caused submaximal effects on the expression of iNOS and the production of NO. Thus, the amount of released nitrite was increased 5.5-fold ($p \leq 0.001$), whereas the transcript level and protein synthesis of iNOS was enhanced 3.5-fold ($p \leq 0.001$) and 282-fold ($p \leq 0.001$), respectively, compared to untreated controls.

Effects of tetracyclines on biosynthesis and activity of iNOS in IL-1α treated articular chondrocytes. Drugs were initially tested at 10 µM in the presence of IL-1α, which reflects the maximum serum level of tetracyclines after therapeutic dosing[22,23]. Those who displayed any significant effects were tested at additional concentrations. Minocycline at concentrations ranging from 1 µM to 100 µM dose dependently inhibited the IL-1 augmented biosynthesis of iNOS (Table 1). IC_{50} values of 69.9 µM and 37.1 µM were determined for the inhibition of mRNA expression and protein synthesis, respectively. However, no reduction of NO production > 32% could be achieved with the maximum concentration of minocycline. On the other hand, no effects on the production and activity of iNOS were observed when chondrocytes were incubated with 10 µM doxycycline and tetracycline, respectively (Table 1).

Effects of NSAID on biosynthesis and activity of iNOS in IL-1α treated articular chondrocytes. Chondrocytes were exposed to diclofenac-Na and meloxicam at a concentration of 10 µM. A significant reduction of released nitrite was observed only in the presence of diclofenac-Na, which also lowered the steady-state level of iNOS mRNA and synthesis of iNOS protein (Table 2). In contrast, meloxicam did not inhibit the accumulation of nitrite in media and solubilized alginate samples. Further, this drug did not significantly affect the expression of iNOS (Table 2).

Effects of tetracyclines and NSAID on the viability and protein synthesis of IL-1α treated articular chondrocytes. The viability of chondrocytes after treatment with IL-1 in the absence or presence of drugs was assessed by the trypan blue exclusion test. Neither the cytokine nor the drugs significantly altered the untreated control values, which were always above 90% viable cells, indicating that tetracyclines and NSAID have no cytotoxic effects on chondrocytes. Furthermore, the addition of these drugs to IL-1 treated chondrocytes did not alter the synthesis of total protein and expression of GAPDH.

DISCUSSION

The involvement of NO in the pathophysiology of OA and RA might present a potential new target for pharmacologic intervention in these diseases. In this respect, the identification of drugs that could efficiently suppress NO production within the joint would be of special interest. Several studies have indicated that tetracyclines such as doxycycline can slow the progression of cartilage damage in animal models of OA[24]. These effects have been mainly attributed to the ability of tetracyclines to inhibit MMP activity and expression[15]. As evidenced by recent studies, though, these drugs can also downregulate the expression of iNOS *in vivo* in mice[25] and *in vitro* in mouse macrophages[26].

We chose chondrocytes because they are among the main producers of NO within the OA affected joint. Accordingly, after exposure to IL-1 we measured a high level of nitrite in the culture supernatants that resulted from increased biosynthesis of iNOS. This IL-1 stimulatory effect was significantly suppressed by minocycline. A comparison of the respective IC_{50} values shows that inhibition was strongest at the level of iNOS protein synthesis, measured during the final 18 h of the treatment period, followed by reduction of mRNA expression and NO (nitrite) production. Whether this indicates that the action of minocycline was time dependent or that minocycline has additional posttranscriptional effects on iNOS remains to be determined. As inhibition of NO production persisted until a concentration of 1 µM, which lies several times under the serum concentration of minocycline[22], the inhibiton of iNOS determined *in vitro* might also occur *in vivo*, and thus might present a new mechanism that could in part explain the improvement seen in RA patients treated with minocycline[14]. However, higher concentrations of minocycline were necessary to reduce NO production by IL-1 treated OA cartilage, indicating either that cartilage might impair diffusion of this drug to chondrocytes or that the putative OA-NOS is differently regulated than iNOS[27].

To date, antidegenerative effects of this drug, and tetracyclines in general, have been mainly explained by their

Table 1. Effects of tetracyclines on NO production, iNOS protein synthesis, and mRNA expression in IL-1 treated articular chondrocytes. Chondrocytes were incubated with IL-1 together with tetracyclines or drug vehicle, respectively. Experiments were performed between culture Day 6 and 8 in the presence of the serum substitute CR-ITS+. (For details see Materials and Methods.)

Condition	Nitrite Released	iNOS Protein Synthesis	iNOS mRNA Expression
IL-1	100	100	100
+1 µM minocycline	91.52 ± 10.1*	118.94 ± 24.1	121.77 ± 24.8
+10 µM minocycline	94.12 ± 6.7*	79.93 ± 16.9*	107.63 ± 20.1
+50 µM minocycline	68.65 ± 12.5**	49.56 ± 16.9**	54.04 ± 20.7**
+100 µM minocycline	68.05 ± 18.2**	22.71 ± 21.9***	46.45 ± 23.4**
*10 µM tetracycline	97.39 ± 18.1	124.54 ± 48.6	106.31 ± 48.0
+10 µM doxycycline	101.87 ± 16.5	111.6 ± 40.6	124.55 ± 28.6

Values are mean ± SD (n = 6). Statistically significantly different from control values (IL-1 + vehicle = 100%): *0.01 < p ≤ 0.05, **0.001 < p ≤ 0.01, ***p < 0.001.

Table 2. Effects of diclofenac-Na and meloxicam on NO production, iNOS protein synthesis, and mRNA expression in IL-1 treated articular chondrocytes. Chondrocytes were incubated with IL-1 together with NSAID or drug vehicle, respectively. Experiments were performed between culture Day 6 and 8 in the presence of the serum substitute CR-ITS+. (For details see Materials and Methods.)

Condition	Nitrite Released	iNOS Protein Synthesis	iNOS mRNA Expression
IL-1	100	100	100
+10 µM diclofenac	91.81 ± 6.1*	89.80 ± 20.3	75.99 ± 22.1*
+10 µM meloxicam	118.52 ± 26.5	127.02 ± 26.6	98.03 ± 15.8

Values are mean ± SD (n = 4–6). Statistically significantly different from control values (IL-1 + vehicle = 100%): *$0.01 < p \leq 0.05$.

inhibitory potential against MMP activity. As there are several reports indicating a role of iNOS in the regulation of MMP activity[4,10], we might hypothesize that downregulation of iNOS is involved in the action by which minocycline inhibits MMP[28] (and data not shown). A possible mechanism could be decreased prostaglandin E_2 (PGE_2) synthesis resulting from the inhibition of NO induced cyclooxygenase-2 expression[29], as reports have shown that PGE_2 can regulate MMP production[30]. Alternatively, NO has been proposed to directly interact with the metal-containing active site of MMP thereby altering the enzyme's configuration and activating MMP from their proenzyme state[4].

Another point for study is the mechanism by which minocycline suppresses iNOS expression. Studies have shown that minocycline inhibits protein kinase C and downregulates cyclic AMP levels[31,32]. However, these signal pathways are not involved in IL-1 induction of iNOS in articular chondrocytes[33]. Further, tetracyclines do not exert their effects on NO production via chelation of Ca^{2+}, as shown in murine macrophages[27]. Thus, the known properties of tetracyclines cannot account for their inhibition of iNOS expression. A possible target of drugs that inhibit iNOS expression could be nuclear factor-κB, which resides in the signal transduction pathway of IL-1 mediated NO induction. Aspirin and dexamethasone, however, which interfere with the transactivating activity of this factor, either inhibit iNOS expression posttranscriptionally (aspirin[34]) or do not affect NO production by chondrocytes (dexamethasone[35]), indicating that evaluating tetracycline's effects on NF-κB might not further our understanding of their effect on iNOS. Two recent publications provide evidence for the involvement of p38 mitogen activated protein kinase[36] and cGMP/phosphodiesterase[37] in IL-1 induction of iNOS in articular chondrocytes. It remains to be determined whether tetracyclines are active in these pathways. Several publications document the inhibition of iNOS expression by aspirin in murine macrophages[18,34]. Recently, this effect has been confirmed in bovine chondrocytes[12]. However, this might not be a common property of NSAID, as these studies also revealed that indomethacin did not inhibit NO production. As NSAID are among the most commonly used drugs in the treatment of OA, the elucidation of differences between members of this class with regard to iNOS inhibition might be helpful in identification of potent inhibitors of inflammation. For this reason, we evaluated the effects of diclofenac-Na and meloxicam on iNOS expression and NO production in articular chondrocytes. At pharmacological concentrations, diclofenac-Na, but not meloxicam, led to a weak reduction in released NO (nitrite) levels, which apparently resulted from decreased mRNA expression of iNOS. Although these results suggest additional antiinflammatory potencies of diclofenac-Na via inhibition of iNOS when administered in experimental models of OA and RA, this drug showed only moderate effects[38,39]. These observations and the fact that the effective concentration of diclofenac-Na in our study lies several times over attainable serum levels[40] indicate that inhibition of iNOS might not occur *in vivo*.

Our study reveals that minocycline inhibits iNOS expression in articular chondrocytes at therapeutic concentrations, suggesting a new mechanism to explain the efficacy of this drug in the treatment of joint diseases.

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