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Preferential inhibition of cyclooxygenase-2 by meloxicam in human rheumatoid synoviocytes

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Abstract

The aim of this study was to evaluate the anti-inflammatory effect of 4-hydroxy-2-methyl-*N*-[5-methyl-2-thiazolyl]-2 *H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide (meloxicam) using cultured rheumatoid synovial fibroblast-like cells (synoviocytes). Synoviocytes were treated with meloxicam in the presence or absence of interleukin-1β. Meloxicam had no effect on both cyclooxygenase-1 and -2 expression as determined by Western blot analysis, immunohistochemical staining, and reverse transcription polymerase chain reaction (RT-PCR). Even the lower doses of meloxicam inhibited cyclooxygenase-2 activity, but only the higher doses of meloxicam inhibited cyclooxygenase-1 activity as determined by prostaglandin E_2 synthesis assay. So meloxicam had a preferential inhibitory effect of cyclooxygenase-2 relative to cyclooxygenase-1 on cultured rheumatoid synoviocytes without affecting cyclooxygenase expression. On the other hand, indomethacin had no selectivity and dexamethasone inhibited the expression of cyclooxygenase-2. Our data indicate that clinical efficacy and safety of meloxicam for rheumatoid arthritis may result from its preferential inhibition of cyclooxygenase-2 activity relative to cyclooxygenase-1 on rheumatoid synoviocytes.

Keywords: Rheumatoid arthritis; Synoviocyte; Cyclooxygenase-1; Cyclooxygenase-2; Meloxicam

1. Introduction

Non-steroidal anti-inflammatory drugs are the most widely used agents for the long-term treatment of musculoskeletal and arthritic syndromes, such as rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis (Baum et al., 1985). They reduce pain, fever, and inflammation, and are also associated with lowered risks of cardiovascular diseases (Willard et al., 1992) and colon cancer (Thun et al., 1991; Rosenberg et al., 1991).

The biological basis for the therapeutic effects of non-steroidal anti-inflammatory drugs is their ability to inhibit prostaglandin biosynthesis. Prostaglandins are lipid mediators whose production is enhanced in both acute and chronic inflammatory reactions (Vane, 1971). It is well known that the mechanism of action of non-steroidal anti-inflammatory drugs is principally by the inhibition of cyclooxygenase, thus blocking the production of proinflammatory prostaglandins (Vane, 1971).

Cyclooxygenase exists in two isozyme forms (Xie et al., 1991; Kujubu et al., 1991) which differ in their basal expression, tissue localization, and induction during inflammation (Vane and Botting, 1996). Cyclooxygenase-1 is a constitutive form of the enzyme that is widely expressed in tissues throughout the body, including the gastrointestinal tract, kidney, and platelets (Smith and De Witt, 1996; Kargman et al., 1996a). The level of expression of the cyclooxygenase-1 gene, as detected by quantitative cyclooxygenase-1 messenger RNA (mRNA) studies, shows little change during the inflammatory process (Crofford et al., 1994). Cyclooxygenase-2, a cytokine-inducible form of the enzyme (Xie et al., 1991; Kujubu et al., 1991), is normally found in very low levels in healthy tissues (except the brain and kidney, where higher constitutive levels are found), but is expressed prominently in inflamed tissues (Kargman et al., 1996a; Crofford et al., 1994;

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Masferrer et al., 1994; Seibert et al., 1994; Vane et al., 1994). The expression of cyclooxygenase-2 varies in magnitude over the course of the inflammatory response (Appleton et al., 1994; Anderson et al., 1996). The induction of cyclooxygenase-2 by inflammatory stimuli, cytokines, or lipopolysaccharides has been demonstrated not only in macrophages (Vane et al., 1994; Fu et al., 1990; Lee et al., 1992; O'Sullivan et al., 1992; Akarasereenont et al., 1995) but also in endothelial cells (Akarasereenont et al., 1995; Habib et al., 1993; Szczepanski et al., 1994) and synovial cells (Crofford et al., 1994; Sano et al., 1992; Knott et al., 1994; Angel et al., 1994). The pharmacology of cyclooxygenase-1 is also different from that of cyclooxygenase-2, such that several non-steroidal anti-inflammatory drugs have been shown to display differential inhibitory activity against cyclooxygenase-2 and cyclooxygenase-1 (Meade et al., 1993; Mitchell et al., 1994).

The analyses of the results obtained with different compounds in different test systems indicate the following trends. Standard non-steroidal anti-inflammatory drugs are either equally effective on cyclooxygenase-1 and cyclooxygenase-2, or slightly more active on cyclooxygenase-1 than on cyclooxygenase-2 (Cromlish and Kennedy, 1996; Churchill et al., 1996; Kargman et al., 1996b; Young et al., 1996; Brideau et al., 1996). 4-Hydroxy-2-methyl-*N*-[5-methyl-2-thiazolyl]-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide (meloxicam) and nimesulide inhibit cyclooxygenase-2 preferentially but not exclusively and some cyclooxygenase-1 inhibitory activity was present in several test systems (Cromlish and Kennedy, 1996; Churchill et al., 1996). Developmental compounds or pharmacological tools such as flosulide, 5-bromo-2[4-fluorophenyl]-3-[(4-methylsulfonyl)phenyl]-thiophene (DUP-697), *N*-[2-cyclohexyloxy-4-nitrophenyl] methanesulfonamide (NS-398), 1-[(4-methylsulfonyl)phenyl]-3-trifluoromethyl-5-[(4-fluoro)phenyl] pyrazole (SC58125), and 5-methanesulfonamido-6-[2,4-difluorothiophenyl]-1-indanone (L-745,337) inhibit cyclooxygenase-2 selectively (Cromlish and Kennedy, 1996; Churchill et al., 1996; Kargman et al., 1996b; Young et al., 1996; Brideau et al., 1996).

Meloxicam is an enolcarboxamide. Minimal changes in the structure of meloxicam alter its affinity and selectivity for cyclooxygenase-2. Changing the methyl substitution from 5′ to the 4′ position on the thiazol group removes the selective inhibition of cyclooxygenase-2 relative to cyclooxygenase-1 (cyclooxygenase-2/cyclooxygenase-1 inhibition ratio ranging from 30–133 in three different assay systems) (Pairet and Engelhardt, 1996).

Recent studies showed that cyclooxygenase-2 levels were increased in ~85% of colorectal adenocarcinomas (Smalley and DuBois, 1997). SC58125 markedly inhibited colorectal tumor growth (Sheng et al., 1997). Meloxicam was also reported to inhibit the growth of the cyclooxygenase-2 positive colorectal cancer cells, but have no effect on the growth of the cyclooxygenase-2 negative cells (Goldman et al., 1998).

Cyclooxygenase-2 in the synovial cells of patients with rheumatoid arthritis plays an important role in the inflammatory process (Sano et al., 1992). In rheumatoid arthritis prostaglandin, E_2 production, which is one of the important mediators of inflammation and joint destruction, is increased. Prostaglandin E_2 derived from cyclooxygenase-2 induces osteoclasts activation and the proliferation of synovial cells as well as endothelial angiogenesis (Hla et al., 1996).

The aim of the present study is to evaluate the anti-inflammatory effect of meloxicam (preferential cyclooxygenase-2 inhibitor) using cultured human rheumatoid synovial fibroblast-like cells (synoviocytes) in comparison with indomethacin (standard non-steroidal anti-inflammatory drug), NS-398 (selective cyclooxygenase-2 inhibitor), and dexamethasone (glucocorticoid).

2. Materials and methods

2.1. Reagents and materials

Collagenase type 1 and acetylsalicylic acid (aspirin) were from Sigma Chemical (St. Louis, MO). RPMI 1640 was from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum and penicillin–streptomycin mixture was from Biowhitterker (Walkersville, MD). Trypsin/EDTA was from GIBCO BRL (Rockville, MD). Meloxicam was obtained from Boehringer Ingelheim Laboratories (Ingelheim, Germany). Dexamethasone and indomethacin were purchased from Wako Pure Chemical (Osaka, Japan). NS-398 was purchased from Cayman Chemical (Ann Arbor, MI).

2.2. Synoviocyte preparation

Rheumatoid synoviocytes were isolated from knee, elbow, and hip joints at the time of total joint replacement surgery in patients with rheumatoid arthritis. All patients fulfilled the 1987 American College of Rheumatology revised criteria for rheumatoid arthritis (Arnett et al., 1988). Synoviocytes were cultured as previously described (Dayer et al., 1976). Briefly, synovia were minced and digested with a mixture of 1 mg/ml collagenase in serum-free RPMI 1640 for 2 h at room temperature. After centrifugation and washing, the cells were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, in a humidified 5% CO_2 atmosphere at 37°C. Non-adherent cells were removed. Adherent cells were cultured in complete medium, trypsinized with trypsin/EDTA at confluence and plated in culture flask (Nunc, Roskilde, Denmark). To obtain a homogeneous population of synoviocytes, confluent cultures from passages 3–7 were used.

2.3. Western blot analysis for cyclooxygenase-1 and cyclooxygenase-2

Adherent cells (2×10^6) were plated in 9.2×1.7 cm of tissue culture dishes (Nunc) and cultured in 10 ml of RPMI 1640 containing 10% fetal bovine serum. Then, the cells were incubated alone or in the presence of 100 μM of meloxicam, indomethacin, NS-398, or 1 μM of dexamethasone, in serum-free RPMI 1640 for 24 h. After supernatants were discarded, 1 ng/ml of interleukin-1β (Otsuka Pharmaceutical, Tokyo, Japan) was added or not and incubated for further 6 h. After incubation, supernatants were harvested and the cells were lysed in 100 μl of solubidization buffer at room temperature (Laemmli sample buffer; Bio Rad, Hercules, CA). Cell lysates were then centrifuged at $13{,}000 \times g$ for 15 min and supernatants were harvested and boiled at 95°C. Aliquots of protein (20 μl) were then separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinilidene difluoride membrane (ATTO, Tokyo, Japan). The membrane was then blocked with phosphate buffered saline (GIBCO BRL) containing 5% skim milk (Difco, Detroit, MI) and 0.2% Tween 20 for 2 h, and incubated with 1:250 of mouse anti-cyclooxygenase-2 monoclonal antibody (Transduction Laboratories, Lexington, KY) or 1:1000 of mouse anti-cyclooxygenase-1 monoclonal antibody (Cayman Chemical) in the blocking buffer at 4°C overnight. The membrane was subsequently incubated with horseradish peroxydase-linked goat anti-mouse immunoglobulin G (IgG) (1:2000 dilution) and analyzed using an Amersham enhanced chemiluminescence system (Amersham Lifescience, Buckinghamshire, England) (Crofford et al., 1994). Hyperfilm (Amersham Lifescience) with cassette closure times of 15–60 seconds resulted in adequate exposure to visualize the bands.

2.4. Extraction of RNA and reverse transcription polymerase chain reaction (RT-PCR) for cyclooxygenase-1 and cyclooxygenase-2

Adherent cells (1×10^7) were plated in 175 cm^3 tissue culture flasks and cultured in 60 ml of RPMI 1640 containing 10% fetal bovine serum for 7 days. Then the cells were incubated alone or in the presence of 100 μM of meloxicam, indomethacin, NS-398, or 1 μM of dexamethasone in serum-free RPMI 1640 for 24 h. After supernatants were discarded, 1 ng/ml of interleukin-1β was added or not and incubated for further 6 h. After incubation, supernatants were harvested and the cells were trypsinized and total RNA was extracted using a modification of the technique as previously described (Chomczynski and Sacchi, 1987), and 200 ng of total RNA was reverse transcribed into complementary DNA (cDNA) (Superscript Preamplification System, GIBCO BRL). The polymerase chain reaction (PCR) was performed with 2 μl of each cDNA, 2 μl of cyclooxygenase-1, cyclooxygenase-2, or glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) primers (20 μM), and 1 unit of Taq DNA polymerase (NIPPON GENE, Toyama, Japan). The oligonucleotide primers were used as follows: cyclooxygenase-1 sense primer 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ and antisense primer 5′-GTGCATCAACACAGGCGCCTCTTC-3′, cyclooxygenase-2 sense primer 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and antisense primer 5′-AGATCATCTCTGCCTGAGTATCTT-3′, G3PDH sense primer 5′-CCACCCATGGCAAATTCCATGGCA-3′ and antisense primer 5′-TCTAGAGGGCAGGTCAGGTCCACC-3′. The primer sets yield PCR products of 303, 304 and 598 bp for cyclooxygenase-1, cyclooxygenase-2, and G3PDH, respectively. Reactions were incubated in an automatic heat-block (Model PJ2000 DNA Thermal cycler, PERKIN ELMER, NJ) for 35 cycles: denaturation 1 min, 94°C; annealing 1 min, 55°C; extension 1 min, 72°C. PCR products were run on 2% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA) and visualized by ethidium bromide staining.

2.5. Immunohistochemical staining of cyclooxygenase-2

Immunoperoxidase staining was performed at room temperature with Vectastain ABC kit (Vector, Burlingame, CA) according to the manufacture's suggested protocol (Hsu et al., 1981; Sano et al., 1990). Adherent cells were plated in LabTek chamber slides (Nunc) at a density of 1×10^5 cells/chamber in 500 μl of RPMI 1640 containing 10% fetal bovine serum at 37°C for 3 days. Then the cells were incubated with or without 100 μM of meloxicam, indomethacin, NS-398, or 1 μM of dexamethasone in serum-free RPMI 1640 for 24 h. After supernatants were discarded, 1 ng/ml of interleukin-1β was added or not and incubated for further 6 h. After incubation, supernatants were harvested and the slides were fixed with acetone for 30 s and immersed in 0.3% peroxidase in methanol for 45 min to deplete endogenous peroxidase. Nonspecific binding sites were saturated with 0.2% bovine serum albumin and normal rabbit serum (Vector) diluted to 1:66.7 in phosphate buffered saline for 20 min. 20 μg/ml of primary antibodies against cyclooxygenase-2 (Santa Cruz Biotechnology, Santa Cruz, CA), or 20 μg/ml of primary antibodies against cyclooxygenase-2 preincubated with 80 μg/ml of synthetic cyclooxygenase-2 (Santa Cruz Biotechnology) for 30 min or normal goat serum (Vector) were applied to the slides and incubated at room temperature for 30 min. After washing in phosphate buffered saline, biotinylated rabbit anti-goat IgG (Vector) in phosphate buffered saline was applied to the slides, and the slides were incubated at room temperature for 30 min. They were then washed in phosphate buffered saline, followed by incubation with avidin and biotinylated horseradish peroxidase complex (Vector) for 45 min. Finally, the slides were immersed in a peroxidase substrate solution containing 0.05% of 3,3′-diaminobenzidine tetra-

hydrochloride (Sigma), 0.04% of nickel chloride (Sigma), and 0.01% of hydrogen peroxide in 0.05 mol/l Tris (pH 7.2) (Wako Pure Chemical) for 5 min. Counter staining was performed with 0.05% light green (Sigma), dehydrated with graded alcohol and xylene for 1 min, respectively, and the coverslides were mounted. Positive staining was indicated as brownish black or dark green deposits on a back ground stain that was light green.

2.6. Cyclooxygenase-1 activity

Adherent cells were cultured in 24 well plates (Nunc) at a density of 2×10^4 cells/well in 500 μl of RPMI 1640 containing 10% fetal bovine serum at 37°C for 3 days. To study the effects on cyclooxygenase-1 activity resting cells (not treated with interleukin-1β) were incubated with meloxicam, indomethacin, NS-398 at various concentrations between 0.01 and 100 μM, or 1 μM of dexamethasone in serum-free RPMI 1640 for 24 h. Then 30 μM of arachidonic acid was added and incubated for further 30 min. After incubation, supernatants were submitted to measurement of prostaglandin E_2 concentration.

2.7. Cyclooxygenase-2 activity

Interleukin-1β stimulated cells were used to study the effects on cyclooxygenase-2 activity. The contribution of cyclooxygenase-1 to total cyclooxygenase activity was suppressed by treating the cells with aspirin (Patrono et al., 1985). Resting cells were cultured with 1 mM of aspirin for 24 h. Then the media were removed and the cells were incubated with meloxicam, indomethacin, NS-398 at various concentrations between 0.01 and 100 μM, or 1 μM of dexamethasone in serum-free RPMI 1640 for 24 h. After supernatants were discarded, 1 ng/ml of interleukin-1β was added and incubated for further 6 h to induce synthesis of cyclooxygenase-2. Then 30 μM of arachidonic acid was added and incubated for further 30 min. After incubation, supernatants were submitted to measurement of prostaglandin E_2 concentration.

2.8. Prostaglandin E_2 synthesis assay

The conditioned media from synoviocytes were collected to measurement of prostaglandin E_2 concentration immediately using a commercially available prostaglandin E_2 monoclonal enzyme immunoassay kit (Assay Designs, Ann Arbor, MI) according to manufacturer's instructions. Prostaglandin E_2 production was evaluated in triplicate serial dilutions of the samples and correlated to a standard curve of prostaglandin E_2.

2.9. Statistical analysis

All results are presented as the mean ± S.E.M. Comparisons of the means were by Fisher's exact test and considered significant when P value is below 0.05.

3. Results

3.1. Effects of meloxicam on the protein expression of cyclooxygenase-1 and cyclooxygenase-2

We examined cyclooxygenase-1 protein expression in synoviocytes at rest, and cyclooxygenase-2 protein in synoviocytes under interleukin-1β stimulated conditions. Total cellular protein was determined by Western blot analysis and immunohistochemical staining. Western blot analysis showed that cyclooxygenase-1 protein expression was identical under any treatment (Fig. 1A). Meloxicam, indomethacin, NS-398, and dexamethasone did not have effects on cyclooxygenase-1 protein expression (Fig. 1A).

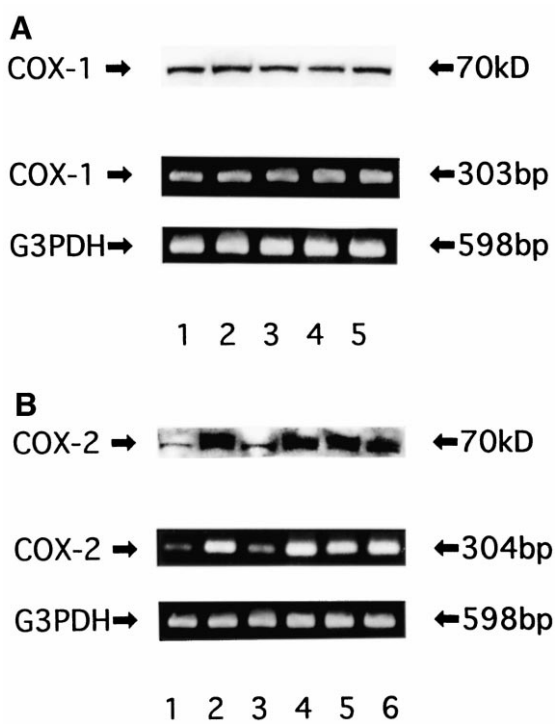

Fig. 1. Cyclooxygenase protein and mRNA expression in meloxicam treated rheumatoid synoviocytes by Western blot analysis and RT-PCR. (A) Cyclooxygenase-1 expression. Aliquots of protein were separated on a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and the filter was incubated with 1:1000 of mouse anti-cyclooxygenase-1 antibody and analyzed by Western blotting as described in Section 2. PCR products were analyzed by electrophoresis on 2% agarose gel as described in Section 2. Lane 1, untreated; Lane 2, dexamethasone (1 μM); Lane 3, meloxicam (100 μM); Lane 4, indomethacin (100 μM); Lane 5, NS-398 (100 μM). Three different experiments were performed ($n = 3$). (B) Cyclooxygenase-2 expression. Aliquots of protein were separated on a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and the filter was incubated with 1:250 of mouse anti-cyclooxygenase-2 antibody and analyzed by Western blotting as described in Section 2. PCR products were analyzed by electrophoresis on 2% agarose gel as described in Section 2. Lane 1, untreated; Lane 2, interleukin-1β (1 ng/ml); Lane 3, interleukin-1β + dexamethasone (1 μM); Lane 4, interleukin-1β + meloxicam (100 μM); Lane 5, interleukin-1β + indomethacin (100 μM); Lane 6, interleukin-1β + NS-398 (100 μM). Three different experiments were performed ($n = 3$).

On the other hand cyclooxygenase-2 protein expression in interleukin-1β stimulated cells was detected, while cyclooxygenase-2 protein expression was little detected in cells at rest (Fig. 1B). Under interleukin-1β stimulated conditions, cyclooxygenase-2 protein expression was suppressed by 1 μM of dexamethasone, but not by 100 μM of meloxicam, indomethacin, and NS-398 (Fig. 1B).

Cyclooxygenase-2 protein was also detected with immunohistochemical method using the antibody against cyclooxygenase-2 in cultured cells (Fig. 2). Under interleukin-1β stimulated condition, cyclooxygenase-2 was expressed in the cytoplasm and nuclear membrane of the cells (Fig. 2B), while cyclooxygenase-2 was little detected in cells at rest (Fig. 2A). Under interleukin-1β stimulated conditions cyclooxygenase-2 expressions were completely negative at control staining with normal goat IgG (Fig. 2C) and anti-cyclooxygenase-2 IgG absorbed with synthetic cyclooxygenase-2 (Fig. 2D). Interleukin-1β-induced cyclooxygenase-2 expression was suppressed by 1 μM of dexamethasone (Fig. 2F), but not by 100 μM of meloxicam (Fig. 2E), indomethacin, and NS-398 (data not shown).

3.2. Effects of meloxicam on the cyclooxygenase-1 and cyclooxygenase-2 MRNA expression

We examined cyclooxygenase-1 mRNA expression in synoviocytes at rest, and cyclooxygenase-2 mRNA in synoviocytes under interleukin-1β stimulated conditions. Total cellular RNA was isolated and RT-PCR was performed. Electrophoresis of PCR products from the cells showed a 303 bp band under any unstimulated condition (Fig. 1A). Cyclooxygenase-1 mRNA expression was identical under

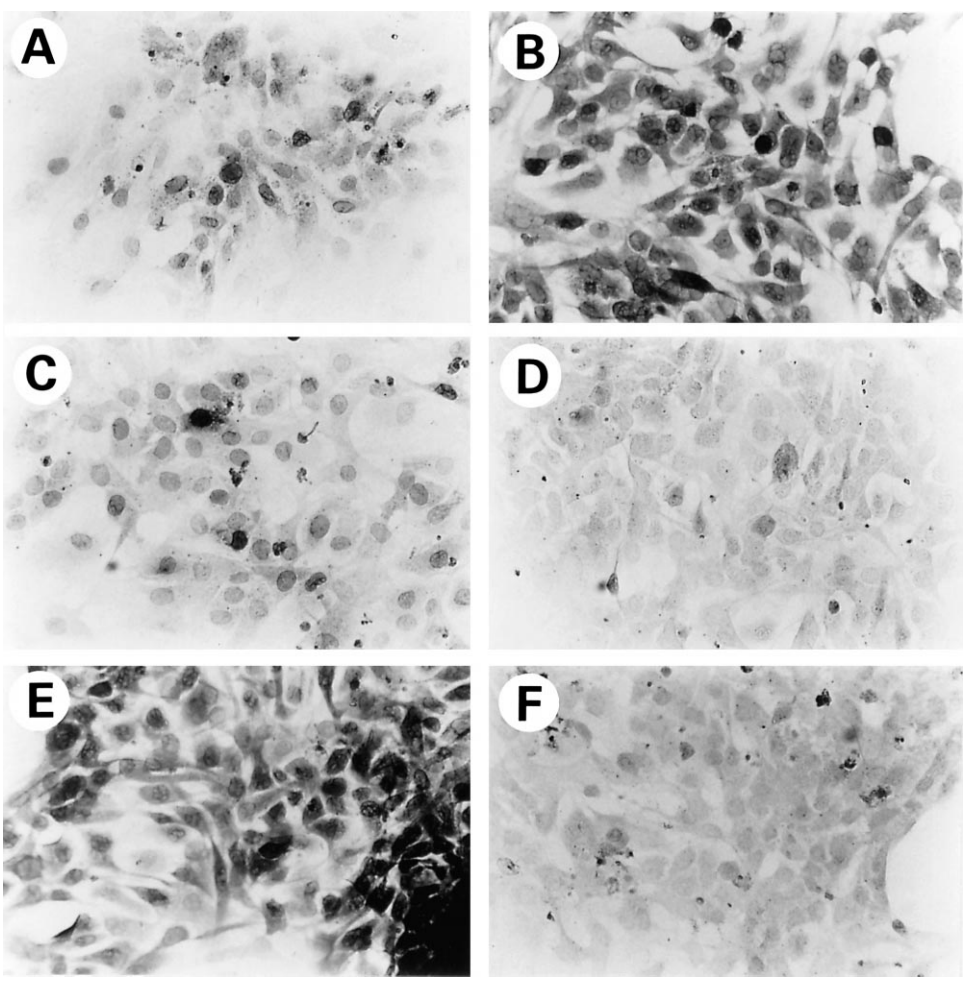

Fig. 2. Cyclooxygenase-2 immunostaining in interleukin-1β stimulated rheumatoid synoviocytes treated with meloxicam. Cultured rheumatoid synoviocytes were stained with goat anti-cyclooxygenase-2 IgG (20 μg/ml) as described in Section 2. Counter staining was done with light green. Positive staining was indicated with brownish black deposits (×100 on original photograph). (A) untreated cells stained with anti-cyclooxygenase-2 IgG, (B) interleukin-1β (1 ng/ml) treated cells stained with anti-cyclooxygenase-2 IgG, (C) interleukin-1β treated cells stained with normal goat IgG (20 μg/ml), (D) interleukin-1β treated cells stained with anti-cyclooxygenase-2 IgG absorbed with synthetic cyclooxygenase-2 peptides, (E) interleukin-1β + meloxicam (100 μM) treated cells stained with anti-cyclooxygenase-2 IgG, (F) interleukin-1β + dexamethasone (1 μM) treated cells stained with anti-cyclooxygenase-2 IgG. Three different experiments were performed ($n = 3$).

any treatment (Fig. 1A). Meloxicam, indomethacin, NS-398, and dexamethasone did not have effects on cyclooxygenase-1 mRNA expression (Fig. 1A).

On the other hand cyclooxygenase-2 mRNA expression in interleukin-1β stimulated cells was detected as a 304 bp band, while cyclooxygenase-2 mRNA expression was little detected in cells at rest (Fig. 1B). Under interleukin-1β stimulated conditions cyclooxygenase-2 mRNA expression was suppressed by 1 μM of dexamethasone, but not by 100 μM of meloxicam, indomethacin, and NS-398 (Fig. 1B).

3.3. Effects of meloxicam on the cyclooxygenase-1 and cyclooxygenase-2 activity

The above results suggest that resting cells express only cyclooxygenase-1, and interleukin-1β stimulated cells express both cyclooxygenase-1 and cyclooxygenase-2. So we precultured the cells with aspirin before adding interleukin-1β to inhibit the cyclooxygenase-1 activity so that we could measure cyclooxygenase-2 activity. As an indicator of cyclooxygenase enzyme activity, we measured prostaglandin E_2 levels in supernatants.

Only the higher doses (>10 μM) of meloxicam significantly inhibited cyclooxygenase-1-derived prostaglandin E_2 production (Fig. 3), while even the lower doses (0.01 μM) of indomethacin inhibited (data not shown). The level

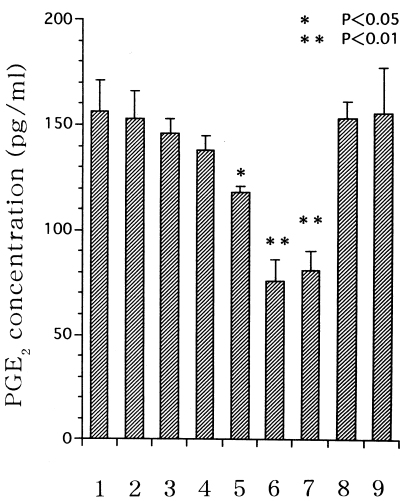

Fig. 3. Effects of meloxicam on the prostaglandin E_2 production by cyclooxygenase-1 in rheumatoid synoviocytes. Cultured rheumatoid synoviocytes were incubated with indicated concentrations of meloxicam, indomethacin, NS-398, or dexamethasone for 24 h. After incubation, the cells were further incubated with 30 μM of arachidonic acid for 30 min. Then prostaglandin E_2 concentrations of the supernatants were quantified by enzyme immunoassay. Lane 1, untreated; Lane 2, meloxicam 0.01 μM; Lane 3, meloxicam 0.1 μM; Lane 4, meloxicam 1 μM; Lane 5, meloxicam 10 μM; Lane 6, meloxicam 100 μM; Lane 7, indomethacin 1 μM; Lane 8, NS-398 100 μM; Lane 9, dexamethasone 1 μM. Values are expressed as means ± S.E.M. of four different experiments ($n=4$) with triplicate samples. * Significant difference from control untreated ($P<0.05$). ** Significant difference from control untreated ($P<0.01$).

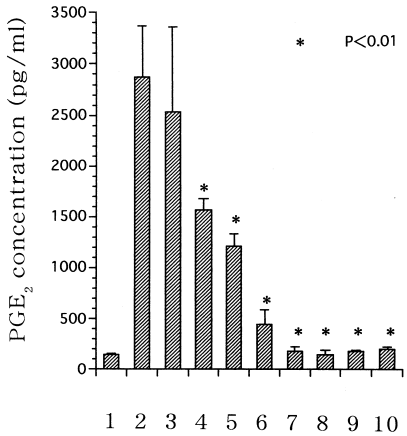

Fig. 4. Effects of meloxicam on the prostaglandin E_2 production by cyclooxygenase-2 in rheumatoid synoviocytes. Rheumatoid synoviocytes were precultured with aspirin (1 mM) for 24 h and incubated with indicated concentrations of meloxicam, indomethacin, NS-398, or dexamethasone for 24 h. After incubation, the cells were stimulated with interleukin-1β (1 ng/ml) for 6 h, and further incubated with 30 μM of arachidonic acid for 30 min. Then prostaglandin E_2 concentrations of the supernatants were quantified by enzyme immunoassay. Lane 1, untreated; Lane 2, interleukin-1β (1 ng/ml); Lane 3, interleukin-1β + meloxicam 0.01 μM; Lane 4, interleukin-1β + meloxicam 0.1 μM; Lane 5, interleukin-1β + meloxicam 1 μM; Lane 6, interleukin-1β + meloxicam 10 μM; Lane 7, interleukin-1β + meloxicam 100 μM; Lane 8, interleukin-1β + indomethacin 100 μM; Lane 9, interleukin-1β + NS-398 100 μM; Lane 10, interleukin-1β + dexamethasone 1 μM. Values are expressed as means ± S.E.M. of four different experiments ($n=4$) with triplicate samples. * Significant difference from control interleukin-1β ($P<0.01$).

of the inhibition of cyclooxygenase-1-derived prostaglandin E_2 production by 100 μM of meloxicam was equivalent to 1 μM of indomethacin (Fig. 3). On the other hand, even the higher doses (100 μM) of NS-398 did not inhibit cyclooxygenase-1-derived prostaglandin E_2 production (Fig. 3); 1 μM of dexamethasone did not, either (Fig. 3).

Prostaglandin E_2 production by interleukin-1β stimulated cells was 20-fold higher than that of untreated (Fig. 4). Meloxicam inhibited interleukin-1β-induced prostaglandin E_2 production in a dose dependent manner (Fig. 4). Even the lower doses of meloxicam significantly inhibited as well as NS-398. The level of the inhibition of cyclooxygenase-2-derived prostaglandin E_2 production by 100 μM of meloxicam was equivalent to that also, caused by 100 μM of indomethacin, NS-398, and 1 μM of dexamethasone (Fig. 4).

4. Discussion

Arthritis and other inflammatory conditions are usually treated with drugs that palliate pain and inflammation. The class of agents most commonly used for this purpose is the non-steroidal anti-inflammatory drugs class. However their use is limited by a significantly increased risk of gastrointestinal ulceration and its complications (such as bleed-

ing and perforation), hemorrhagic diathesis, and nephrotoxicity (Rodriguez and Jick, 1994; Gabriel et al, 1991; Langman et al., 1994; Schafer, 1995; Palmer, 1995). It is now well established that cyclooxygenase-2 mediates the production of prostaglandins associated with inflammation and pain (Masferrer et al., 1994; Seibert et al., 1994; Portanova et al., 1996), whereas vital homeostatic prostaglandins are produced by cyclooxygenase-1 (Masferrer et al., 1994; Seibert et al., 1994; Smith and De Witt, 1996). Toxicity of non-steroidal anti-inflammatory drugs is therefore considered to result primarily from inhibition of cyclooxygenase-1.

Meloxicam is a new non-steroidal anti-inflammatory drug which preferentially inhibits cyclooxygenase-2 relative to cyclooxygenase-1, as consistently demonstrated in a number of models (Pairet et al., 1998; Ogino et al., 1997; Blanco et al., 1999). Preclinical studies have demonstrated that meloxicam is a potent anti-inflammatory agent in animal models of acute inflammation and arthritis (Engelhardt et al., 1995). Meloxicam significantly inhibits carageenan-induced paw oedema in the rat and both the primary and secondary responses in the rat adjuvant-induced arthritis model (Engelhardt et al., 1995). The ratio of the ulcerogenic potential of meloxicam compared with the anti-inflammatory activity shows that meloxicam has a superior therapeutic index compared with other standard non-steroidal anti-inflammatory drugs tested. Furthermore, clinical trials have demonstrated a favorable gastrointestinal safety profile for meloxicam (Fenn and Morant, 1996; Hawkey et al., 1998; Lipscomb et al., 1998; Lemmel et al., 1997).

These studies support the hypothesis that preferential inhibition of cyclooxygenase-2 by meloxicam may contribute to the improved therapeutic index and safety profile that has been observed for meloxicam in vivo. Furthermore, there are other differences in the pharmacodynamic profile of meloxicam in comparison with other non-steroidal anti-inflammatory drugs. Anti-inflammatory doses of meloxicam do not influence leukotrien C4 content of tissue and have no influence on bronchial tone. That means that the risk of bronchoconstriction may be lower with meloxicam than with known non-steroidal anti-inflammatory drugs. Efficacy and safety of meloxicam in patients with rheumatoid arthritis had already been reported by clinical trials (Lemmel et al., 1997), but the study directly using synoviocytes from patients with rheumatoid arthritis has not been performed yet.

In the present study, we directly evaluated using cultured rheumatoid synoviocytes the anti-inflammatory effect in comparison with standard non-steroidal anti-inflammatory drug and selective cyclooxygenase-2 inhibitor. We examined whether the higher doses of meloxicam inhibited cyclooxygenase-1 and cyclooxygenase-2 expression. Meloxicam did not inhibit cyclooxygenase-1 expression, both at protein and mRNA level as well as indomethacin, NS-398, and dexamethasone. Furthermore, meloxicam did not inhibit cyclooxygenase-2 expression, both at protein and mRNA level as well as indomethacin and NS-398, while dexamethasone inhibited.

Moreover we used prostaglandin E_2 assay system to measure the inhibitory effects on cyclooxygenase-1 and cyclooxygenase-2 activity of meloxicam compared with indomethacin, NS-398, and dexamethasone. Cyclooxygenase-1 activity determined by the prostaglandin E_2 concentration of non-stimulated rheumatoid synoviocytes was significantly inhibited by only the higher doses of meloxicam. On the other hand, indomethacin inhibited cyclooxygenase-1 activity even at the lower doses, and NS-398 did not inhibit even at the higher doses. Cyclooxygenase-2 activity, determined by the prostaglandin E_2 concentration of precultured with aspirin and interleukin-1β stimulated rheumatoid synoviocytes, was dose-dependently inhibited by meloxicam. Even the lower doses of meloxicam inhibited as well as NS-398. And the inhibitory effect was equivalent with indomethacin and NS-398. Non-steroidal anti-inflammatory drugs including meloxicam inhibited cyclooxygenase activity without inhibition of expression, while glucocorticoid inhibited cyclooxygenase expression. These results suggest that meloxicam preferentially inhibits cyclooxygenase-2 activity relative to cyclooxygenase-1 on cultured rheumatoid synoviocytes without inhibition of cyclooxygenase expression.

Non-steroidal anti-inflammatory drugs could be useful for the treatment of inflammatory processes associated with Alzheimer's disease (Fiebich et al., 1996). Meloxicam slightly inhibit the interleukin-1β induced synthesis of interleukin-6 in a human astrocytoma cells as well as naproxen, whereas ibuprofen, piroxicam, diclofenac, and indomethacin have no effects (Fiebich et al., 1996). In rheumatoid synoviocytes meloxicam had no significant inhibitory effect of the interleukin-1β-induced synthesis of interleukin-6 (data not shown). Meloxicam has inhibitory effect on the growth of the cyclooxygenase-2 positive colorectal cancer cells (Goldman et al., 1998). In rheumatoid synoviocytes, meloxicam had no inhibitory effect of proliferation (data not shown). But meloxicam may have other unknown effects.

In conclusion, our data indicate that clinical efficacy and safety of meloxicam for rheumatoid arthritis may result from its preferential inhibition of cyclooxygenase-2 activity relative to cyclooxygenase-1 on rheumatoid synoviocytes.

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