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Methotrexate as a preferential cyclooxygenase 2 inhibitor in whole blood of patients with rheumatoid arthritis

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Abstract

Objective. To investigate the regulation of whole-blood cyclooxygenase-1 and -2 (COX-2 and COX-1) activities by methotrexate (MTX) in rheumatoid arthritis (RA) patients.

Methods. Whole blood was withdrawn from nine healthy volunteers, 12 RA patients treated with MTX (RA/MTX) and six RA patients treated with chloroquine (RA/CQ). COX-1 activity was quantified as platelet thromboxane B_2 production in unstimulated blood and COX-2 activity was measured as prostaglandin E_2 (PGE_2) production in whole blood stimulated with LPS. Thromboxane B_2 and PGE_2 were measured by radioimmunoassay. We studied the drug effect *in vitro* by direct incubation of MTX with blood obtained from normal donors. *Ex vivo* assays were performed with blood collected from RA/MTX and RA/CQ patients. The influence of serum factors on enzyme activities was analysed in blood collected from normal donors and incubated with RA/MTX, autologous or heterologous serum.

Results. *In vitro* assays showed no direct action of MTX on the activity of either enzyme. Assays performed with blood from RA/MTX patients showed preferential inhibition of COX-2 activity ($PGE_2 = 10.11 \pm 2.42$ ng/ml) when compared with blood of normal donors ($PGE_2 = 37.7 \pm 4.36$ ng/ml; $P = 0.001$). Inhibition of COX-2 activity was also observed when blood of normal donors was co-incubated with RA/MTX serum.

Conclusion. Our results clearly show that the anti-inflammatory action of low-dose MTX is partly mediated by a serum factor induced by MTX or a MTX metabolite that preferentially inhibits the activity of COX-2.

KEY WORDS: Methotrexate, Anti-inflammatory agents, Cyclooxygenases, Rheumatoid arthritis.

Rheumatoid arthritis (RA) is an autoimmune disease in which inflammation of the cells lining the synovium produces pain, swelling and progressive erosion of the synovial joints. Cyclooxygenase (COX) is an enzyme that catalyses the conversion of arachidonic acid to prostaglandin H_2, which is further converted to other prostaglandins, prostacyclin and thromboxanes [1, 2]. There are two COX isozymes, called COX-1 and COX-2, encoded by separate genes. COX-1 is a constitutive enzyme and mediates functions such as the regulation of kidney functions, stomach acid secretion, and inhibition of platelet aggregation [2]. The COX-2 isozyme mediates pain [3] and is induced mainly during the inflammatory process by monocytes stimulated by cytokines and mitogens [2, 4].

Recent results from animal models and clinical studies suggest that COX-2 is up-regulated in inflammatory joint disease and is a potential therapeutic target in arthritis [5]. Rabbits with antigen-induced arthritis exhibit an increased prostaglandin E_2 (PGE_2) level in synovial fluid, which is reduced by previous administration of dexamethasone [6]. Immunocytochemical studies of synovial membrane from patients with RA and rats with adjuvant arthritis, using a COX-2-specific antibody, showed positive staining in RA patients, but not in synovial membrane from normal subjects [5].

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Additionally, synovial cells from RA patients produced large amounts of PGE_2 and also showed increased gene expression of COX-2, but not of COX-1 [7].

Very limited information is available about the effects of disease-modifying anti-rheumatic drugs (DMARDs) on the activity of COX enzymes. It remains unclear how low-dose methotrexate (MTX) acts as an anti-inflammatory agent. Recent results obtained in our laboratory showed that, like dexamethasone, MTX decreases PGE_2 and interleukin-1 levels in synovial fluid collected from rabbits with arthritic knees [6]. This study was designed to test, *in vitro* and *ex vivo*, the effectiveness of MTX as an inhibitor of COX-1 and COX-2 activities in the whole blood of rheumatoid arthritis patients.

Patients and methods

Patient selection for the ex vivo assay

Peripheral whole blood for measurement of COX-2 and -1 activity was collected from 15 patients with RA (12 women and three men, aged 57.13 ± 3.28 yr). All patients met the American College of Rheumatology 1987 revised criteria [8]. Written informed consent was obtained from them before they entered the study. The mean duration of the disease was 11.14 ± 2.65 yr. Clinical evaluation was performed at each visit. Pain, duration of early morning stiffness, number of swollen and/or tender joints, and Westergren sedimentation rate were quantified and combined in an activity score as described by Wilke *et al.* [9] (0 = no activity, 8 = maximal activity). No patient had received concomitant therapy with non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticosteroids within 2 weeks before the study. Twelve patients had a regimen of 5–7.5 mg MTX taken as a single weekly oral dose (RA/MTX patients). Three patients were without medication at study entry. Nine healthy volunteers who were sex- and age-matched served as normal controls. All refrained from taking NSAIDs for at least 2 weeks before donating blood. Additionally, six RA patients treated with 250 mg chloroquine daily (RA/CQ patients) were studied; they were similar to the patients treated with MTX with respect to age (53 ± 4.01 yr) and disease duration (8.3 ± 2.15 yr). All steps for the measurement of whole-blood COX-2 and COX-1 activities in the patients and control were performed as described below under the heading *In vitro* assays.

In vitro assays

Measurement of COX-1 activity in human whole blood. Methotrexate (Lederle-Cyanamid, Wolfrats-house, Germany) was previously diluted in phosphate-buffered saline (PBS) and 2 μl of each solution was added to the vials. The final concentrations of MTX in the assay were 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M, and a control tube was incubated with 2 μl PBS. Normal human venous blood was collected with no anticoagulant, and 500 μl was immediately added to each tube containing 2 μl test substance. The tubes were vortexed and placed in an incubator at 37°C for 60 min. Serum was separated by centrifugation (10 min at 3000 r.p.m.) and 100 μl was removed and added to a tube containing 400 μl methanol. After another centrifugation, supernatant was kept at −70°C until assayed for thromboxane B_2 [10].

Measurement of COX-2 activity in human whole blood. Human fresh venous blood was collected with no anticoagulant and 500 μl was immediately added to each tube containing 2 μl test substance, heparin (10 IU/ml), lipopolysaccharide from *Escherichia coli* (LPS; 100 μg/ml). To assess the basal activity of the enzyme, a control tube was incubated in the absence of LPS. The tubes were vortexed and placed in an incubator at 37°C for 24 h. Plasma was separated and PGE_2 extracted and quantified by the same procedure as that described for thromboxane B_2 (TxB_2) [10].

Influence of serum collected from RA/MTX patients on whole-blood COX-2 and COX-1 activities. In order to assess the effects of serum factors on COX-2 and COX-1 activities, 250 μl blood from each of six healthy subjects was co-incubated with 250 μl serum collected from six RA/MTX patients as well as with autologous or heterologous serum. The assays for COX-1 and COX-2 activities were performed as described above and the results corrected for the dilutions performed.

Analyses of TxB_2 and PGE_2. Concentrations of PGE_2 and TxB_2 were measured by radioimmunoassay using commercial kits (DuPont/New England Nuclear NEN™, Boston, MA, USA) [10].

Statistical analysis

Results are expressed as mean ± S.E.M. A probability level of <0.05, as assessed by analysis of variance followed by the Newman–Keuls test, was considered significant.

Results

In vitro effect of MTX on COX-2 and COX-1 activities

The *in vitro* effects of MTX on COX-1 and COX-2 activities were quantified by measurement of PGE_2 and TxB_2 in the serum of six normal, healthy donors exposed to different drug concentrations. The results of *in vitro* assays showed no direct action of any dose of MTX on the activity of either enzyme (COX-1: 290.21 ± 12.86 ng/ml TxB_2 for MTX, 284.46 ± 46.76 for controls; COX-2: 32.03 ± 1.81 ng/ml PGE_2 for MTX, 30.46 ± 2.69 for controls).

Ex vivo evaluation of whole-blood COX-2 and COX-1 activities in RA/MTX patients

The results of the analysis of whole-blood COX-2 and COX-1 activities in 12 RA/MTX patients, six RA/CQ patients and nine matched normal controls are presented in Fig. 1. Our results clearly show that the use of MTX by RA patients promoted preferential inhibition of COX-2 activity. RA patients treated with CQ showed PGE_2 values within the normal range, similar to those obtained with whole blood collected from normal donors and significantly different from those obtained in

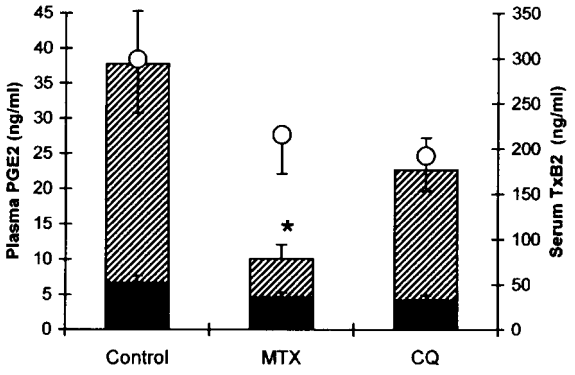

FIG. 1. Prostaglandin E_2 production in heparinized whole blood stimulated with LPS for 24 h as a reflection of COX-2 activity of monocytes (▨) and basal activity of COX-2 in unstimulated monocytes (■). Thromboxane B_2 (TxB_2) production in whole blood incubated for 1 h as an indicator of COX-1 activity (○). The *ex vivo* assay was performed with blood collected from 12 RA/MTX patients (MTX), six RA/CQ patients (CQ) and nine normal healthy donors (Control). Values are mean ± S.E.M. $^*P < 0.05$.

RA/MTX patients. The basal plasma concentrations of PGE_2 (without LPS) was not affected by any treatment (4.65 ± 0.9 ng/ml PGE_2 for RA/MTX patients, 4.23 ± 0.56 ng/ml for RA/CQ patients and 6.7 ± 1.03 ng/ml for normal donors). Inhibition of whole-blood COX-1 activity was not found in RA/MTX patients or in patients treated with CQ. No correlation was found between the Wilke activity score (3.33 ± 0.61) and COX-2/COX-1 activities. It should be emphasized that the numbers of circulating monocytes and platelets in all groups of patients were within the normal range, and did not correlate with the level of COX-1 or COX-2 activity (data not shown).

Influence of serum factors on COX-1 and COX-2 activities

In order to investigate the influence of serum factors on enzyme activity, whole blood was collected from normal donors and exposed to RA/MTX, autologous or heterologous serum during the stimulation of COX-1 and COX-2 activity. Results in Table 1 indicate that specific inhibition of COX-2 activity was obtained only when blood from normal donors was co-incubated with RA/MTX serum. The co-incubation with autologous or heterologous serum did not interfere with enzyme activity. No inhibitory influence of RA/MTX serum on COX-1 activity was detected.

TABLE 1. Effect of serum on whole-blood COX-2 and COX-1 activities

Normal donors	RA/MTX serum	Autologous serum	Heterologous serum
Whole-blood PGE_2 production stimulated with LPS COX-2 activity			
6	29.75 ± 2.28*	42.5 ± 4.66	40.25 ± 3.6
Whole-blood thromboxane B_2 production COX-1 activity			
6	184.6 ± 23.3	233.75 ± 17.5	176.25 ± 17.4

Blood from six normal donors was co-incubated with RA/MTX serum, autologous serum or heterologous serum. Data are expressed as mean ± S.E.M.

$^*P < 0.05$ compared with autologous and heterologous values.

Discussion

The aim of this study was to test the effect of low-dose methotrexate on COX-2 and COX-1 activities using a whole-blood assay [10]. This assay simulated the physiological responses of humans to drug administration, thereby resulting in a more accurate *ex vivo* measurement of the ability of a drug to inhibit COX-2 and COX-1 activities [10, 11, 12, 13]. We tested the *in vitro* action of MTX by incubation of blood collected from normal donors with the drug, and the *ex vivo* effect was studied using blood from RA patients treated only with MTX. Our results showed a reduction of whole-blood COX-2 or CQ activity when the assay was performed with blood collected from RA patients treated with a low dose of methotrexate. RA patients treated with CQ showed PGE_2 production within the normal range, similar to the result obtained with whole blood from normal donors. It is worth noticing that the concentration of PGE_2 in the whole blood of three RA patients without medication was similar to that in blood from normal donors (33.52 ng/ml). Furthermore, we found no correlation between prostaglandin levels and the Wilke activity score. COX-1 activity was not altered by MTX or CQ therapy.

The results of the *in vitro* assay performed with different concentrations of MTX showed no direct action of the drug on whole-blood COX-2 or COX-1 activities. This result probably rules out a direct action of the drug on either enzyme.

In order to investigate the influence of serum factors on enzyme activity, whole blood collected from normal donors was exposed to RA/MTX, autologous or heterologous serum during assays for COX-1 and COX-2 activities. The results showed a preferential ability of serum from RA/MTX patients to inhibit COX-2 activity. This inhibition of COX-2 activity, verified only in the presence of RA/MTX serum, supports a direct effect on the enzyme, possibly mediated by some inhibitory mediator induced by this drug or an MTX metabolite. Cronstein *et al.* [14] and Morabito *et al.* [15] have linked MTX to the inhibition of amino imidazole carboxamidonucleotide transformylase and the subsequent accumulation of adenosine, a potent inhibitor of neutrophil function. Results obtained previously in our laboratory showed an effect of a metabolite of MTX on neutrophil superoxide production [16].

Thus, MTX induces the presence of an inhibitory factor or an MTX metabolite, which inhibits COX-2 activity without affecting COX-1 activity, as suggested by the following observations. First, whole blood from RA/MTX patients showed a reduction in the concentration of PGE_2 after LPS stimulation. Secondly, co-incubation of serum from these RA/MTX patients with blood from healthy donors promoted preferential

inhibition of COX-2 activity without affecting the concentration of TxB_2 (COX-1 activity). In order to confirm this hypothesis we performed the *ex vivo* COX-1 and COX-2 activity assays using blood collected from a non-RA patient treated only with MTX (5 mg/week) for psoriasis. The results showed an inhibitory action of MTX specifically on whole-blood COX-2 activity. In this patient, the production of PGE_2 was reduced by the same magnitude as that observed in RA/MTX patients (8 ng/ml), and the production of TxB_2 was within the normal range (232.5 ng/ml). The *ex vivo* down-regulation of whole-blood COX-2 activity by MTX could explain its early-onset anti-inflammatory action during the treatment of RA patients.

Our results clearly show that part of the anti-inflammatory action of low doses of MTX is mediated by a serum factor induced by MTX or a metabolite of MTX that preferentially inhibits the activity of COX-2 without affecting COX-1 activity.

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