

Superoxide production by polymorphonuclear leucocytes in rheumatoid arthritis and osteoarthritis: in vivo inhibition by the antirheumatic drug piroxicam due to interference with the activation of the NADPH-oxidase

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SUMMARY The superoxide (O_2^-) production of stimulated polymorphonuclear leucocytes is increased in patients with rheumatoid arthritis and osteoarthritis compared with controls. Treatment of these different groups with pharmacological amounts of the non-steroidal anti-inflammatory drug piroxicam in vivo resulted in a decrease of about 25% in O_2^- secretion by isolated granulocytes. In vitro experiments showed that piroxicam inhibits O_2^- production of granulocytes by interference with the stimulation of the NADPH-oxidase. Piroxicam caused diminished O_2^- production of membrane fragments if it was present during the stimulation of the NADPH-oxidase of the intact cells. During the actual O_2^- production of the stimulated membrane fragments piroxicam had no effect. It is concluded that piroxicam is able to inhibit granulocyte O_2^- production by blocking the activation of NADPH-oxidase, which results in diminished tissue destruction by oxygen free radicals in inflammatory diseases.

Key words: granulocyte inhibition, mechanism of NSAIDs, granulocyte membrane fragments, superoxide anion, phagocytosis.

The importance of oxygen free radicals and related activated oxygen intermediates in the pathogenesis of rheumatoid arthritis (RA) is increasingly recognised.^{1–4} In RA and in other inflammatory diseases polymorphonuclear leucocytes (PMN) and macrophages are stimulated, which results in the secretion of inflammatory mediators, including large amounts of superoxide (O_2^-) and hydrogen peroxide (H_2O_2).⁵ These cells produce O_2^- by the NADPH-oxidase, an enzyme complex located in the plasma membrane and phagosome. The reduction equivalents to reduce oxygen to O_2^- are derived from NADPH. In the non-phagocytosing cell the

NADPH-oxidase is inactive. When the cell is stimulated to phagocytose an activation mechanism for NADPH-oxidase is triggered. Little is known about the molecular basis of this activation mechanism. These activated oxygen intermediates together with secondary formed radicals, like the hydroxyl radical (OH^\cdot), are able to destroy membrane lipids, proteins, deoxyribonucleic acid, hyaluronic acid, and cartilage.^{3,6,7} Previous investigations have shown that different antirheumatic drugs are able to inhibit the production of oxygen free radicals.^{8–10} Thus it is reasonable to believe that non-steroidal anti-inflammatory drugs (NSAIDs) have a beneficial effect not only by the inhibition of cyclooxygenase, as shown by the excellent work of Vane,¹¹ but also by preventing the formation of oxygen free radicals.

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Until now the inhibition of the O_2^- producing NADPH oxidase by NSAIDs has been mostly tested in vitro. This study is focused on the in vivo inhibition of O_2^- production from PMN by piroxicam, which was chosen because of the encouraging results already obtained.¹²⁻¹⁴ O_2^- production of PMN was tested before and at different times after the administration of piroxicam to patients with RA and osteoarthritis, and to controls. Attention was also given to the molecular level at which piroxicam interferes with the NADPH-oxidase.

Patients and methods

PATIENTS

PMN were isolated at different times from patients with classical or definite RA, from patients with osteoarthritis, and from healthy volunteers. Patients on D-penicillamine, gold salts, or corticosteroids were excluded. All other drugs were discontinued three days before administering piroxicam. If necessary paracetamol was allowed.

Piroxicam was given orally, 40 mg at time zero and another 20 mg 24 h later. When blood was taken for PMN isolation a serum sample was also drawn, and the serum piroxicam was measured by high performance thin layer chromatography.¹⁵ Before and during the observation period no other NSAIDs were taken.

PMN ISOLATION

PMN were harvested from defibrinated blood by sodium metrizoate-Ficoll centrifugation (Lymphoprep, Nyegaard & Co., Oslo, Norway). Erythrocytes were lysed by a cold isotonic NH_4Cl solution.¹⁶ The final cell suspension contained >95% PMN. At least 95% of the cells were viable, based on the exclusion of trypan blue.

CAPACITY OF PMN TO PRODUCE O_2^-

Isolated PMN were measured for their capacity to produce O_2^- by the cytochrome *c* reduction method. The isolated cells were resuspended in a solution containing: 137 mM NaCl, 5.4 mM KCl, 0.7 mM NaH_2PO_4 , 0.8 mM $MgSO_4$, 1.3 mM $CaCl_2$, 5.5 mM glucose, 25 mM trometamol (TRIS) (pH 7.4 at 37°C), and 5 g/l of bovine serum albumin. The incubation mixture contained in final concentrations: 0.7×10^6 PMN/ml ($0.7 \times 10^9/l$) exactly, 150 μM cytochrome *c*, and as stimulus 100 $\mu g/l$ phorbol myristate acetate (PMA) or 1 g/l serum treated zymosan. After 15 min at 37°C absorption at 550 nm was measured. The O_2^- production was calculated from the increase of the absorption by using an extinction coefficient of 21 100 litre $mol^{-1} cm^{-1}$ for cytochrome *c*. Control experiments showed that

cytochrome *c* reduction was completely blocked by the addition of 13 mg/l superoxide dismutase.

For measurements of the effect of piroxicam in vitro PMN were obtained from healthy individuals. Cells were resuspended in: 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 1 mM $MgCl_2$, 0.6 mM $CaCl_2$, pH 7.4 (PiCM buffer). Incubations were performed with 3.0×10^6 PMN/ml ($3.0 \times 10^9/l$). Piroxicam (Pfizer BV Rotterdam, The Netherlands) was dissolved in 1 M NaOH, followed by correction of the pH, to final concentrations of 25, 50, and 100 $\mu mol/l$. After 5 min preincubation (37°C) 150 μM cytochrome *c* and 200 $\mu g/l$ PMA were added (final concentrations). At different times samples were drawn and O_2^- production was measured.

O_2^- PRODUCTION BY MEMBRANE FRAGMENTS

Membrane fragments were isolated from control persons according to Tauber and Goetzl.¹⁷ Isolated PMN were preincubated with or without 100 μM piroxicam for 5 min. This was followed by stimulation of the cells by incubation with 1 mg/l PMA for three minutes at 37°C. Cells were homogenised at 0°C and differential centrifugation followed. O_2^- production was measured in a mixture containing: PMN membrane fragments 10 μg protein/ml (mg/l) in PiCM buffer, 1 mM NADPH, and 150 μM cytochrome *c*. The absorbance was measured at 550 nm in a double beam spectrophotometer. The effect

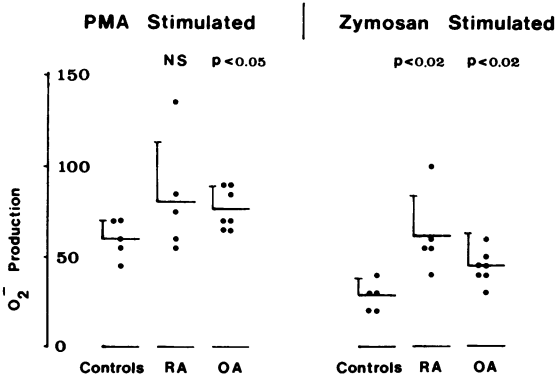


Fig. 1 O_2^- production by stimulated PMN from: controls ($n=5$), patients with rheumatoid arthritis (RA) ($n=5$), and patients with osteoarthritis (OA) ($n=7$). Mean and SD of O_2^- production are expressed as $nmol/10^6$ PMN in 15 minutes. PMA or serum treated zymosan was used as stimulus. Significant differences with the control group are indicated. NS=not significant.

of the addition of 100 μM piroxicam during the O_2^- production was also studied. All measurements were duplicated.

Results

O_2^- PRODUCTION OF PMN ISOLATED FROM PATIENTS WITH UNTREATED RA AND OSTEOARTHRITIS, AND FROM CONTROLS At two different times with an interval of 24 h PMN were isolated from blood from patients with RA and osteoarthritis, and from healthy controls, before treatment with piroxicam. Without stimulation of the cells only very small amounts of O_2^- were

secreted, without difference between the groups. The O_2^- production after stimulation with either PMA or serum treated zymosan is presented in Fig. 1. The mean production of the two different PMN isolations from each individual is shown. After stimulation with PMA no significant difference in O_2^- production was found between RA patients and controls. In contrast, patients with osteoarthritis produced 129% O_2^- in comparison with controls ($p < 0.05$). After stimulation with serum treated zymosan O_2^- secretion was significantly higher in both RA and osteoarthritis patients and equalled 212% ($p < 0.02$) and 154% ($p < 0.02$) respectively.

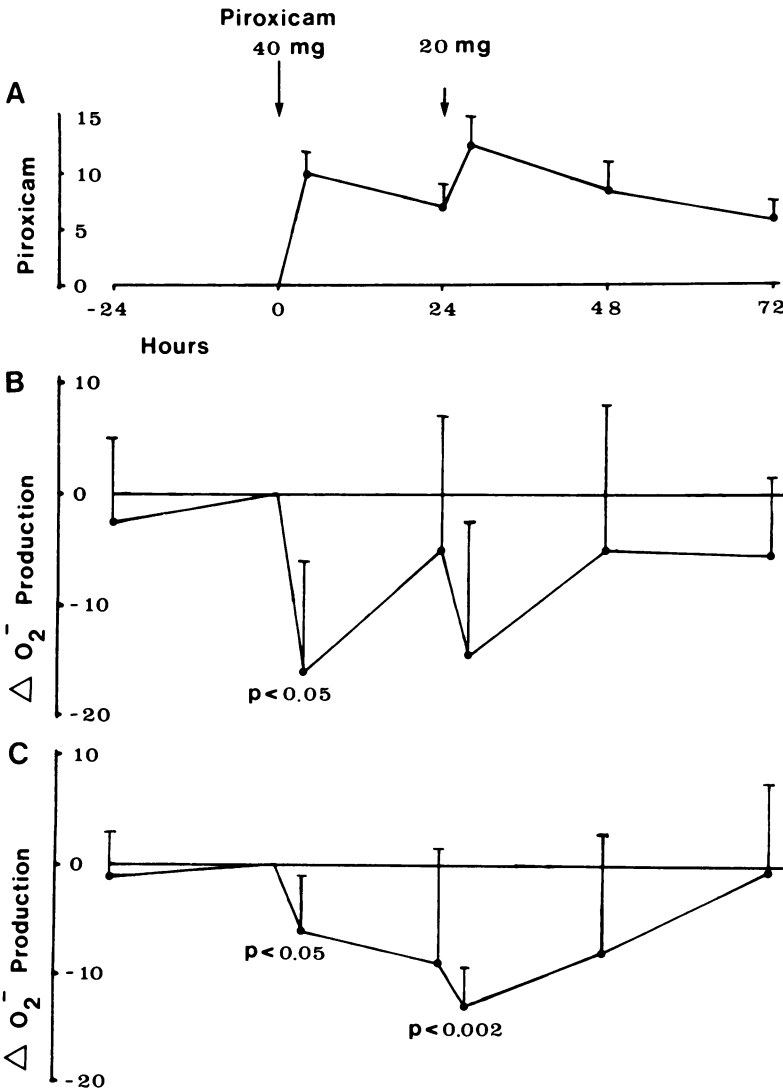


Fig. 2 The effect of piroxicam administered to control persons (n=5) on the O_2^- production by stimulated PMN isolated at different times. (A) Piroxicam concentration in $\mu\text{mol/l}$; (B) O_2^- production of PMN stimulated with PMA; (C) O_2^- production of PMN stimulated with serum treated zymosan. O_2^- production (in $\text{nmol}/10^6$ PMN in 15 minutes) is expressed as the difference in the O_2^- production at the indicated time and at time zero. Mean and SD are shown. Significant differences are indicated.

O₂⁻ PRODUCTION OF PMN ISOLATED AFTER TREATMENT WITH PIROXICAM IN VIVO
 The effect of administering piroxicam orally on the O₂⁻ production of PMN isolated after treatment was measured in RA and osteoarthritis patients and in healthy controls. Piroxicam was taken orally at two times, 40 mg at time zero and another 20 mg 24 h later. At the times indicated PMN were isolated

and O₂⁻ production after stimulation with PMA or serum treated zymosan was determined (Figs 2, 3, and 4). After taking the blood samples PMN were washed five times so that no serum containing piroxicam was left during the O₂⁻ estimation. The isolation procedure took about 2½ h. For each individual the difference in O₂⁻ production at various times was expressed in comparison with the

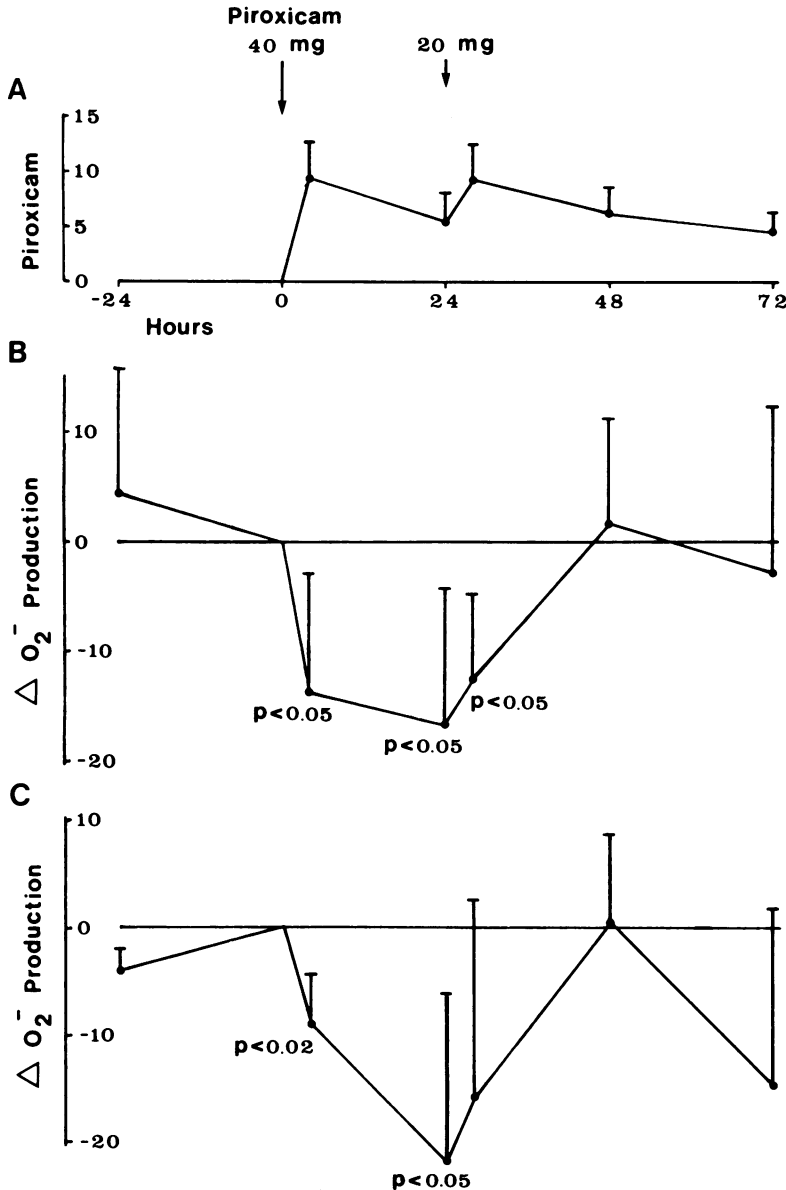


Fig. 3 The effect of piroxicam administered to RA patients (n=5) on the O₂⁻ production by stimulated PMN isolated at different times. For further explanation see legend to Fig. 2.

O_2^- production at time zero, immediately before the first dose of piroxicam. This presentation was used to diminish the effect of variation in O_2^- production before piroxicam treatment.

There was again very little O_2^- production without stimulation of the cells with PMA or serum treated zymosan and this was not changed by piroxicam treatment (data not shown). In the control group (n=5), (Fig. 2) there was a significant decrease in O_2^- production four hours after taking 40 mg piroxicam, which was independent of PMA or

serum treated zymosan as stimulus. At 28 hours, four hours after the second dose of piroxicam (20 mg), the O_2^- production was only significantly decreased when serum treated zymosan was used as stimulus. At 48 and 72 hours the O_2^- production approached the level before treatment. The data at t=-24 hours indicate the variation of the O_2^- production without treatment.

The RA patients (n=5) (Fig. 3) showed similar results. Again piroxicam treatment induced a significant decrease in O_2^- production between four and 28 hours, which was followed by a return to the basal O_2^- production at 48 hours.

Patients with osteoarthritis (n=7) (Fig. 4) showed the same pattern, but the decrease in O_2^- production was significant only at 28 hours.

No significant change in O_2^- production was found in controls without piroxicam treatment in a period of four days, and no diurnal variation was found.

The effect of piroxicam was essentially the same in all different groups. Treatment with piroxicam resulted in an average decrease in O_2^- production of 25%. It should be noted that owing to the extensive washing during the cell isolation, no serum containing piroxicam was left during the O_2^- measurements. It can be concluded that piroxicam treatment in vivo changed the ability of isolated PMN to produce O_2^- .

LEVEL OF INTERACTION OF PIROXICAM WITH THE NADPH-OXIDASE OF PMN

Fig. 5 shows that the inhibition of O_2^- production of intact PMN by piroxicam was dependent on concentration. To identify the mode of inhibition of the

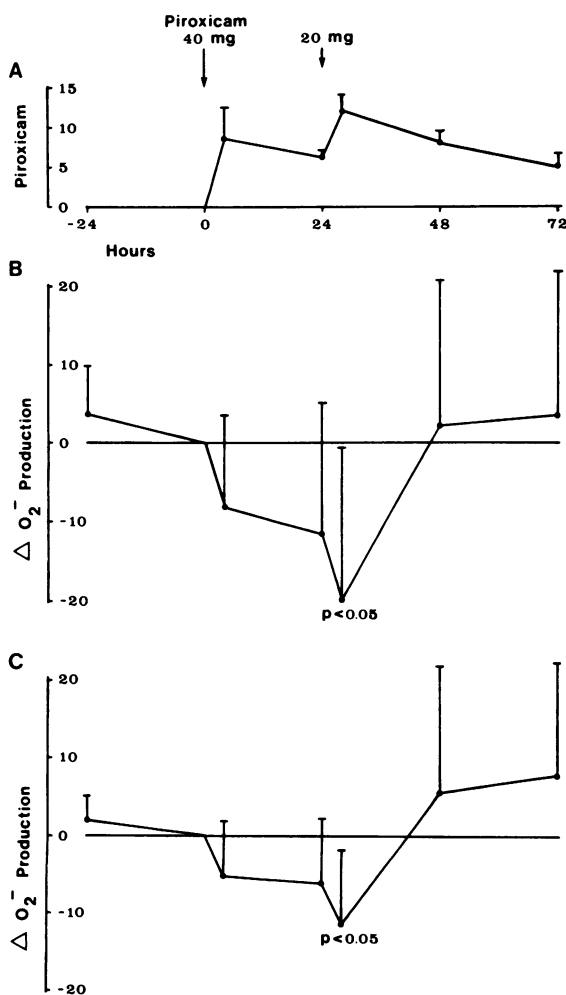


Fig. 4 The effect of piroxicam administered to osteoarthritis patients (n=7) on the O_2^- production by stimulated PMN isolated at different times. For further explanation see legend to Fig. 2.

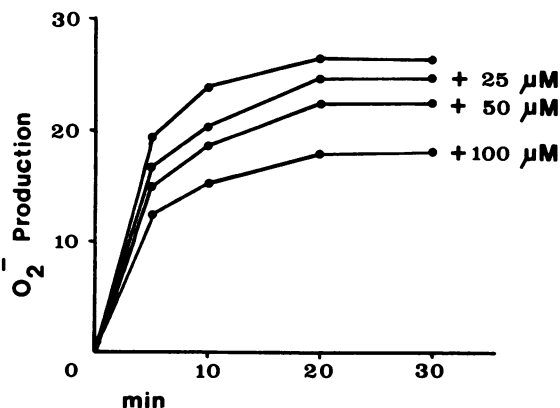


Fig. 5 The in vitro inhibition of O_2^- production (nmol/10⁶ PMN in 15 minutes) of PMN stimulated by PMA incubated in presence of various piroxicam concentrations.

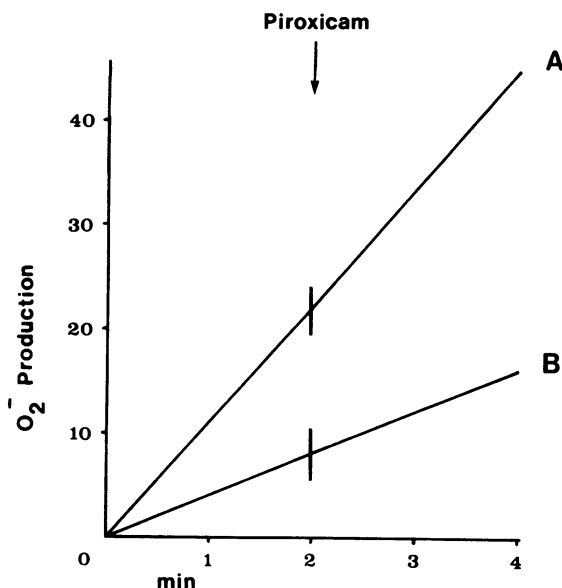


Fig. 6 O_2^- production (nmol/mg protein) by membrane fragments of PMN stimulated by PMA. During stimulation of the NADPH-oxidase of the intact PMN $100 \mu M$ piroxicam was absent in A and present in B. The effect of $100 \mu M$ piroxicam during the O_2^- production is also shown (addition indicated by an arrow).

O_2^- producing NADPH-oxidase complex PMN were homogenised after stimulation of the NADPH-oxidase with PMA. The capacity of membrane fragments to produce O_2^- was not changed by the addition of $100 \mu M$ piroxicam during the O_2^- production (Fig. 6). Membrane fragments obtained from PMN stimulated with PMA in the presence of piroxicam produced smaller amounts of O_2^- in comparison with fragments of cells stimulated in the absence of piroxicam. Addition of piroxicam to these membrane fragments did not result in enhanced inhibition. These results indicate that piroxicam has no effect on the NADPH-oxidase complex itself if it is already stimulated, but that piroxicam interferes in the mechanism by which the NADPH-oxidase is stimulated.

Discussion

The exact role of oxygen free radicals in the pathogenesis of RA is not yet established. However, many authors are convinced that these radicals have an important role.¹⁻⁴ Phagocytosing granulocytes are known to produce considerable amounts of O_2^- and H_2O_2 .⁵ In juvenile RA stimulated granulocytes are shown to produce twice as much O_2^- as control

PMN.¹⁸ No difference in O_2^- production was found between adult RA patients and controls,¹⁷ but the study of Chiu *et al.* showed that PMN from patients with Felty's syndrome produced only 63% O_2^- compared with the controls.¹⁹ The present investigations show that after stimulation with serum treated zymosan PMN from patients with RA or osteoarthritis produce an increased amount of O_2^- compared with controls. With PMA stimulated PMN the increase was only significant in the osteoarthritis group. This increased potency of PMN to produce O_2^- together with the availability of stimulating factors for PMN in serum and synovial fluid of RA patients²⁰ will result in considerable O_2^- production in vivo. In the presence of non-protein bound iron, possibly derived from ferritin,²¹ formation of the very toxic hydroxyl radical has to be expected, and tissue destruction will result.

The discovery of the inhibition of prostaglandin synthesis by NSAIDs, due to inhibition of cyclooxygenase, indicated an important mechanism of action of NSAIDs. At this moment, however, it seems very likely that in addition other mechanisms have a role in the beneficial effect of these drugs. Previous investigations have shown that NSAIDs like indomethacin, ibuprofen, phenylbutazone and piroxicam⁸⁻¹² are able in vitro to decrease the release of O_2^- from PMN. Abramson *et al.*^{13 14} showed that piroxicam did also inhibit O_2^- production of PMN in vivo in healthy volunteers. In four RA patients no significant effect was found.²² In these studies O_2^- production was measured only at two times—before and during treatment with piroxicam. The present study shows the effect of two doses of piroxicam, and O_2^- production was measured at seven different times—twice before treatment to show the natural variation, at different times during the phase of high piroxicam serum levels, and during the recovery phase.

The results show that treatment of patients with piroxicam caused diminished O_2^- production of their PMN. The average decrease was 25%. In contrast with the studies of Abramson *et al.*^{13 14 22} we found not only significant decrease in O_2^- production in normals but also in RA and osteoarthritis patients. For the different groups: RA patients, osteoarthritis patients, and controls, no essential differences in the inhibition patterns were found. During the recovery phase the O_2^- production showed a tendency to normalise. In vivo PMN probably are inhibited to a larger extent, because the isolation procedure of the PMN lasted for some time and involved frequent washing of the cells. These manipulations will possibly decrease the effect of piroxicam on the cells. The conclusion can be drawn that in vivo piroxicam is able to decrease

the O_2^- production of PMN to a considerable extent.

To obtain information about the mechanism of inhibition investigations were performed on the level at which piroxicam inhibited the O_2^- production of PMN. Isolated membrane fragments were used, which are known to contain the O_2^- producing NADPH-oxidase. Stimulation of the NADPH-oxidase is only possible in intact cells, possibly with the exception of stimulation with *cis* unsaturated fatty acids.²³ For this reason cells were homogenised after stimulation of the NADPH-oxidase with PMA, followed by isolation of the membrane fragments. The O_2^- production of the PMA stimulated NADPH-oxidase of membrane fragments decreased if piroxicam was present during the activation of the NADPH-oxidase in the intact cells. Addition of piroxicam to the isolated membrane fragments had no effect on the O_2^- production. It can therefore be concluded that piroxicam interferes in the mechanism by which the NADPH-oxidase is activated. For this reason piroxicam can potentially be very useful for investigations designed to elucidate the mechanism by which NADPH-oxidase is stimulated.

Overall it seems reasonable to believe that piroxicam, and possibly other NSAIDs, cause improvement in RA and osteoarthritis not only by inhibition of prostaglandin synthesis but also by decrease of the O_2^- production of PMN and macrophages, which results in the prevention of tissue destruction caused by oxygen free radicals.

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