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Inhibitory and enhancing effects of piroxicam on whole blood chemiluminescence

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ABSTRACT: The effects of piroxicam on the production of reactive oxygen species by stimulated phagocytes was studied in whole blood by a chemiluminescence (CL) technique in relation to maximum activity, localization and kinetics of radical generation. We found that piroxicam dose-dependently inhibited total (intra- and extracellular) zymosan-stimulated luminol CL (LCL) at a high stimulant concentration ($p = 0.0001$). Piroxicam additionally decreased cytochalasin B-reduced LCL, which shows that the effect of the drug should be sought in the extracellular component of the response. Piroxicam inhibited the first phase of extracellular LCL in a dose-dependent manner ($p = 0.0001$) and revealed itself as an enhancing agent of CL in later time intervals after the start of respiratory burst, in a model system containing horseradish peroxidase (HRP) and sodium azide. It enhanced LCL of a cell-free system, i.e. influenced the CL due to HRP-catalysed decomposition of hydrogen peroxide. It also dose-dependently inhibited the early extracellular superoxide production, evaluated by lucigenin CL ($p = 0.022$). Piroxicam inhibited the total fMLP-stimulated LCL by 70% approximately and, only by about 30%, the first phase of fMLP-stimulated extracellular LCL, which presupposes an effect on myeloperoxidase-catalysed formation of hypochloric acid. Piroxicam slightly increased the intracellular LCL by phagocytes ($p = 0.02$), an effect that is probably connected with its ability to induce the release of secondary messengers in signal transduction. In conclusion, the anti-inflammatory effect of piroxicam is probably related to the inhibition of the extracellular generation of superoxide and hypochloric acid in the early stages of phagocyte activation. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: piroxicam; peripheral leukocytes; reactive oxygen species; CL; extracellular

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are therapeutic agents used for treatment of individuals with disturbances accompanied by pain, fever or inflammation. The inhibition of the prostaglandin system through blocking cyclooxygenase is a widely accepted mode of action of these compounds. Over the years, many authors have described a number of non-prostaglandin-mediated anti-inflammatory effects, particularly effects on different stages of the leukocyte activation cascade (1–3). Recently, some pro-oxidant properties of NSAIDs have also been reported (4–6).

Piroxicam is a NSAID of the oxicam family. Except for being an inhibitor of cyclooxygenase-1, it shows an anti-inflammatory effect through decreasing neutrophil aggregation and chemotaxis in response to various stimuli (1, 7). Piroxicam increases leukocyte plasma membrane viscosity, which presupposes some changes in the post-receptor signalling events (2). It should be mentioned that the effect of piroxicam on some leukocyte functions *in vitro* could be observed only at drug concentrations much higher than the therapeutic plasma concentrations (8).

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A number of studies with some of their results being controversial have examined the effect of piroxicam on the oxidative activity of stimulated leukocytes using a CL technique. Thus, for instance, it has been reported that piroxicam inhibits phorbol myristate acetate and N-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated luminol CL (LCL) by isolated neutrophils (9, 10). On the other hand, an increase in LCL by zymosan-stimulated isolated neutrophils has been established, which the authors relate to some pro-oxidant activity of the drug (11). According to Colli *et al.* (12), piroxicam *in vitro* added to whole blood reduces superoxide generation induced by fMLP, A 23187 and serum-treated zymosan. The cause of the differences observed may be due to the different methodology used, stimulant and experimental conditions. Many studies were also carried out with isolated leukocytes but it is known that the cellular isolation procedure affects oxidant generation and enzyme release, activates and primes leukocytes, changes the proportion between different leukocyte subpopulations, and, thus, may cause a misinterpretation of results (13). Furthermore, it is not always clear whether the drug effect detected is on extra- or intracellularly generated radicals, especially with the CL technique (14). It is known that generated reactive oxygen species (ROS) released in excess into the extracellular space can damage the surrounding cells.

It is therefore very important to discriminate between radicals of extra- and intracellular localization.

The aim of the present study was to evaluate the effect of piroxicam on the kinetics of extracellular, intracellular and total ROS generation by stimulated peripheral phagocytes in whole blood.

MATERIALS AND METHODS

Reagents

The stock solutions of luminol (10^{-2} mol/L) and lucigenin (10^{-2} mol/L) in dimethyl sulphoxide (DMSO) were stored in a dark place at room temperature and diluted 1:10 with Krebs–Ringer phosphate buffer (KRP) immediately prior to use. Zymosan-A was opsonized for 30 min at 37°C in normal pooled human serum (20 mg/mL), washed twice in 0.9% NaCl and lyophilized. The stock zymosan suspension (6 mg/mL in KRP) was prepared immediately prior to use. The stock solution of fMLP (1 mg/mL in DMSO) was prepared before use and diluted with KRP to a concentration of 2×10^{-5} mol/L at the intermediate stage. The stock solution of cytochalasin B (2 mg/mL in DMSO) was prepared before use and diluted with KRP to an intermediate concentration of 100 μg/mL. The stock solutions of horseradish peroxidase (40 U/mL), superoxide dismutase (3000 U/mL), catalase (30 000 U/mL) and sodium azide (0.1 mol/L) were prepared before use. Piroxicam was dissolved in DMSO to a stock solution of 2 mg/mL and diluted with KRP to the corresponding final concentrations. It was incubated with blood for 5 min at 37°C. We consider that a comparison of leukocyte functions in the presence and in the absence of piroxicam could be properly made for relatively small times of incubation, since, according to literature, piroxicam affects cellular aggregation (15, 16). The range of the concentrations used varied from 8 μg/mL (25.5 μmol/L) to 80 μg/mL (255 μmol/L), i.e. they exceeded the therapeutic plasma concentrations. A reason for that was: (a) there are data published suggesting that the concentration of some NSAIDs in knee cartilage *ex vivo* exceeds that in the surrounding medium and peripheral blood (17); (b) it has been reported that piroxicam affects some stages of leukocyte activation cascade at high concentrations only (8). All the reagents used were purchased from Sigma-Aldrich (St Louis, MO, USA).

Blood parameters

Blood from healthy individuals anti-coagulated with heparin (10 U/mL) was used. Each time the total number of leukocytes, erythrocytes and differential cell counts were determined.

CL evaluation of ROS generation

The phagocyte activity to produce ROS was studied in whole blood. The CL kinetics, reflecting this activity were registered by a computerized chemiluminometer (18). Luminol and lucigenin were used as CL amplifiers. Zymosan A and fMLP were used as stimulating agents of leukocytes. All components of the sample with the exception of diluted whole blood were preliminary thermostated in the luminometer cuvettes at 37°C for 5 min. After adding the blood, the contents of the cuvettes were mixed and LCL kinetics of six samples were simultaneously registered for 20 min.

Luminol CL (LCL)

Evaluation of total whole blood LCL (total LCL). The standard system contained 0.1 mL whole blood (1:10), luminol (10^{-5} mol/L), opsonized zymosan (0.25 mg/mL or 4 mg/mL) or fMLP (5×10^{-7} mol/L) and KRP in a total volume of 2 mL (19). LCL of the system represents the total ROS generation by phagocytes (extra- and intracellular), which is myeloperoxidase (MPO)-dependent.

Evaluation of extracellular whole blood LCL (LCL of cytochalasin B-treated whole blood). Cytochalasin B (Cyt B) was used as an inhibitor of actin polymerization in the phagocyte cytoskeleton, i.e. as an inhibitor of phagocytosis (20, 21). That is why LCL in the presence of Cyt B is of an extracellular origin. The system, besides the components of the standard system, also contained Cyt B (5 μg/mL).

Evaluation of extracellular whole blood LCL (extra LCL). The system used, besides the components of the standard system, contained also 1 mmol/L NaN_3 (an extracellular and intracellular MPO inhibitor) and 4 U/mL horseradish peroxidase (HRP; an azide-insensitive enzyme added instead of the extracellular MPO) (22). NaN_3 inactivates MPO and HRP regenerates CL since LCL is a peroxidase-dependent one. HRP is a large molecule that does not readily gain access to the intracellular sites and, hence, the CL registered by this system is of an extracellular origin. To analyse the enhancing effect of piroxicam on the second phase of extra LCL, exogenous superoxide dismutase (SOD) (150 U/mL) was added in the sixth minute after the start of zymosan-stimulated response.

Evaluation of intracellular whole blood LCL (intra LCL). The system used, besides the components of the standard system, also contained SOD (150 U/mL) and catalase (CTS) (1500 U/mL) (23). SOD and CTS are large molecules, which do not gain access to the intracellular sites and, thus, remove only the

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extracellularly localized superoxide and hydrogen peroxide. That is why LCL of this system is of an intracellular origin.

LCL of cell-free system

Piroxicam effect on LCL generated in a model cell-free system was studied in order to check whether it changes the CL produced by HRP-catalysed decomposition of H_2O_2. The system contained HRP (0.2 U/mL), H_2O_2 (0.35 nmol/mL) and luminol (10^{-5} mol/L). In this case, piroxicam and luminol concentrations were the same as in the cellular systems. The reason for that was to avoid a misinterpretation of the results, since there are data published that piroxicam changes the fluorescence of some compounds (15). The concentration of hydrogen peroxide used was of the same order of magnitude as that produced by zymosan-stimulated phagocytes under our experimental conditions, which we evaluated according to Nahum *et al.* (24). HRP concentration was reduced as compared to that used in the cellular systems, since otherwise the CL generated was too high and the response was totally completed within 1 min.

Lucigenin CL (LgCL)

It was used to evaluate piroxicam effect on the extracellular superoxide generation (25). The samples contained 0.1 mL whole blood (1:10), lucigenin (10^{-5} mol/L), zymosan (4 mg/mL) and KRP in a total volume of 2 mL.

Since under some experimental conditions bimodal chemiluminescent kinetics were registered and the drug affected the separate phases differently, it was inconvenient to evaluate its effect by the parameter 'area under the CL kinetic curve'. In most cases, the effect of piroxicam was evaluated by the parameter 'maximum LCL intensity' (I_{max}) of the corresponding kinetic phase, which represents the maximum achieved leukocyte activity to produce ROS. In some experiments, depending on the aim of the analysis, the effect of the drug was evaluated in the first minute after the start of the response.

Statistical analysis

The inhibitory/activatory effect of piroxicam on the chemiluminescent intensity was computed according to the equation:

$$\%\ \text{inhibition/activation} = \frac{|I_{\text{piroxicam}} - I_{\text{control}}|}{I_{\text{control}}} \times 100$$

where $I_{\text{piroxicam}}$ and I_{control} are the chemiluminescent intensities (in the peak or in the first minute) in the presence or absence of piroxicam, respectively. The results were analysed by Kruskal–Wallis ANOVA and median test, since they deviated from Normal distribution. When the effect of the drug was found significant (H-value and the corresponding level of significance p are given in the text), Mann–Whitney U test was applied to determine the exact difference between the groups studied (the level of significance p is given in the corresponding figures). Data are presented as median (minimum–maximum value).

RESULTS AND DISCUSSION

Piroxicam effect on ROS production by stimulated phagocytes was studied using a CL technique. The changes arising in the oxidative response of peripheral phagocytes with respect to magnitude, localization and kinetics of ROS generation were investigated.

Piroxicam dose-dependently inhibits total LCL at a high zymosan concentration

It is considered that phagocytes are the main source of light in whole blood total CL. This luminescence is an expression of a complex series of events non-uniformly distributed among different cells, as well as on the surface of a single cell. At the same time, the light produced is a result of simultaneous ROS generation of extra- and intracellular localization.

According to the literature, piroxicam interferes with the exchange of GTP/GDP by regulatory G proteins in the neutrophil plasmalemma (26). This is why we stimulated the leukocytes by opsonized zymosan particles. It is known that opsonized zymosan activates the respiratory burst of leukocytes by F_c and C_{3b} receptors, which belong to the family of G-protein-coupled receptors.

We found that the effect of piroxicam on the total light generation (total LCL) depended on the stimulant concentration (Fig. 1A). The drug dose-dependently inhibited LCL intensity ($H = 19.6$; $p = 0.0001$) when the cells were strongly stimulated (zymosan 4 mg/mL) (Fig. 1B). At a low zymosan concentration (0.25 mg/mL), a tendency to an increased LCL response was observed (Fig. 1A). In our previous study, on the basis of a theoretical model component analysis of the CL kinetics, we reported that zymosan concentration substantially changes the relative contribution of extra- and intracellular light generation within the total response—from an almost missing extracellular portion at a low zymosan concentration to a markedly predominant extracellular component at a high zymosan concentration (27). Hence, the different effect of piroxicam observed may be due to the fact that the drug influences in a different way the extracellular and intracellular light whose relative contribution within the total response is different at different stimulant concentrations. Since the initial cellular activation has been considered to reflect

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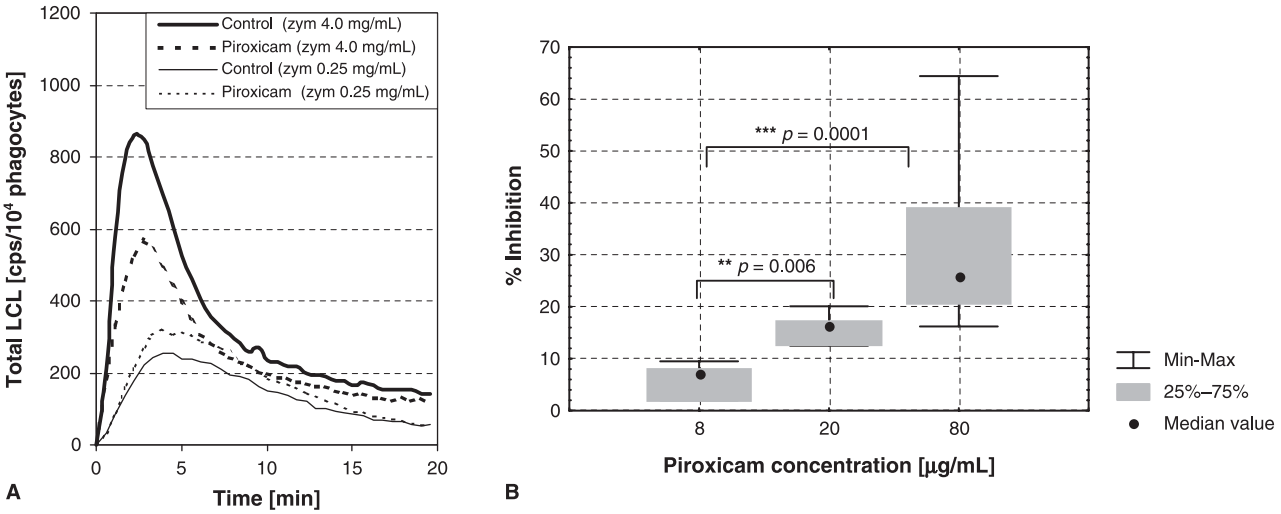

Figure 1. (A) Effect of piroxicam (80 µg/mL) on total LCL at two different concentrations of opsonized zymosan; representative tracings. (B) Inhibitory effect of piroxicam on total LCL intensity registered in the first minute after the start of response (zymosan 4.0 mg/mL). Number of measurements: n (8 µg/mL) = 7; n (20 µg/mL) = 7; n (80 µg/mL) = 14.

the extracellular ROS generation (28, 29), the inhibitory effect of piroxicam at the high stimulant concentration was computed for total LCL intensity, registered in the first minute after the start of the response (Fig. 1B).

Piroxicam inhibits zymosan-stimulated extracellular LCL by cytochalasin B-treated whole blood

In confirmation of the supposition that piroxicam affects extracellular CL, we studied its effect on LCL by phagocytes in the presence of Cyt B.

It is known that Cyt B is an inhibitor of actin polymerization. It uncouples the membrane receptors from the cytoskeleton and greatly reduces the respiratory burst activity of leukocytes (20, 21). Since Cyt B inhibits the particle uptake, ROS generation takes place mainly on the leukocyte surfaces (21). It was found that Cyt B (5 µg/mL) markedly diminished whole blood LCL response at a high zymosan concentration [median 70.4% (minimum value 57.8%, maximum value 75.6%)] (Fig. 2) and almost fully eliminated the response at a low stimulant concentration (data not shown). We also found that piroxicam additionally inhibited the Cyt B-reduced LCL at the high zymosan concentration [median 39.4% (minimum value 28.8%, maximum value 55.3%)] (Fig. 2). Hence, the inhibitory effect of piroxicam should be sought in the extracellular portion of total LCL response.

Effect of piroxicam is different on the separate phases of bimodal zymosan-stimulated extracellular LCL (extra LCL)

Piroxicam effect on the extracellular LCL was studied in a model system generating mainly extracellular light.

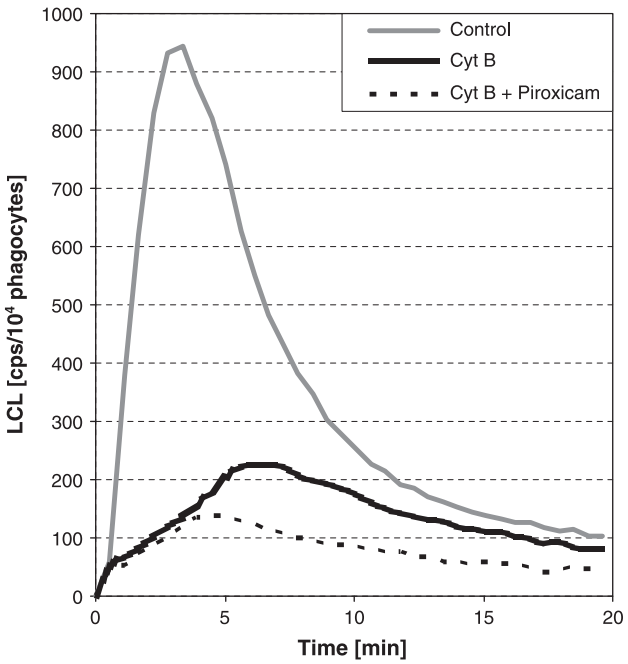

Figure 2. Effect of piroxicam (80 µg/mL) on LCL of Cyt B-treated whole blood (Cyt B 5 µg/mL; zymosan 4.0 mg/mL); representative tracings of five experiments.

For that purpose, the system used contained sodium azide (which inhibits MPO) and exogenous horseradish peroxidase (which regenerates LCL) (Fig. 3A). It was found that piroxicam inhibited I_{max} of the first phase of the kinetics in a dose-dependent manner (H = 19.53; p = 0.0001) (Fig. 3B). In the extracellular system, the same quantity of horseradish peroxidase was always added at blocked MPO. Therefore, it could be considered that piroxicam exerted a dose-dependent inhibition of extracellular superoxide generation in the initial stages of phagocyte activation.

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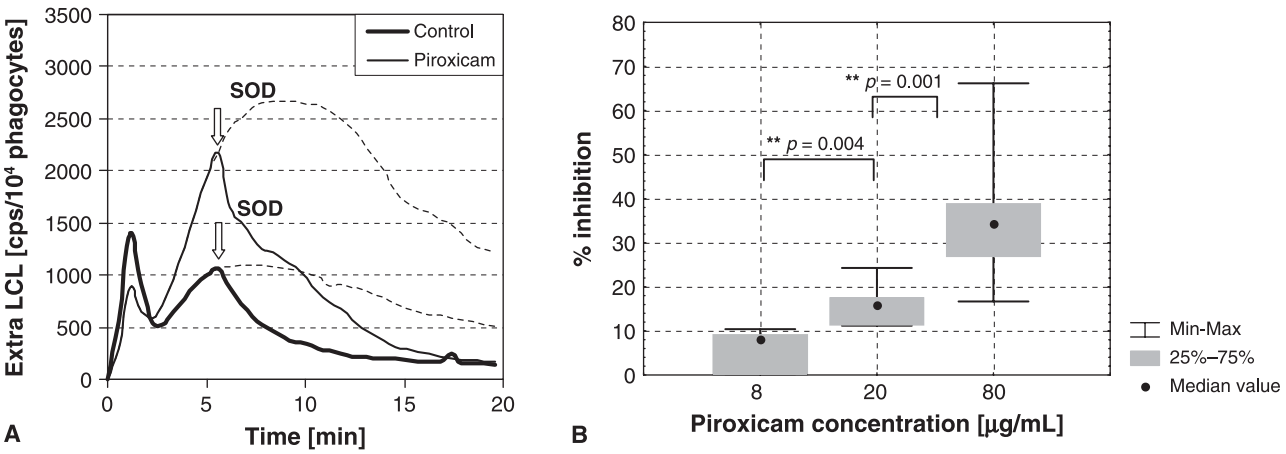

Figure 3. (A) Effect of piroxicam (80 µg/mL) on extra LCL (zymosan 4.0 mg/mL); representative tracings. SOD (150 U/mL) was added to the contents of the extracellular model system in the sixth minute after the start of response. (B) Dose-dependent inhibitory effect of piroxicam on the maximum LCL intensity of the first phase of extra LCL. Number of measurements: n (8 µg/mL) = 7; n (20 µg/mL) = 7; n (80 µg/mL) = 14.

On the other hand, piroxicam substantially increased the second phase of extra LCL, independently of concentration ($H = 3.65$; $p = 0.16$). In the literature, HRP-mediated oxidation of biomolecules, such as ascorbate, nicotinamide adenine dinucleotide and glutathione, in the presence of NSAIDs, has been reported. It has also been shown that some NSAIDs metabolize to ROS by activated neutrophils (5, 6). It is therefore possible for the enhancing activity of the drug to be due to the accelerated oxidation of some biomolecules by piroxicam radicals. In this case, however, since the exogenous SOD reduced extra LCL in a similar way in the presence and absence of piroxicam (Fig. 3A), the drug most probably changes the activity of HRP.

Piroxicam enhances LCL produced by cell-free model system

We studied the effect of piroxicam on LCL, generated in a cell-free system containing HRP and H_2O_2 to check whether its activating effect on extra LCL was due to some interaction with the system HRP–H_2O_2. An activating effect of piroxicam on the maximum LCL intensity produced by this system was observed. The effect was more pronounced at lower piroxicam concentrations ($H = 8.71$; $p = 0.01$) (Fig. 4). Some additional data are needed to ascertain whether piroxicam increases the peroxidase-catalysed decomposition of H_2O_2.

Piroxicam inhibits fMLP-stimulated total LCL and has a different effect on the separate phases of fMLP-stimulated extracellular LCL (extra LCL)

Total LCL by stimulated phagocytes depends on the quantity of both the superoxide produced and the

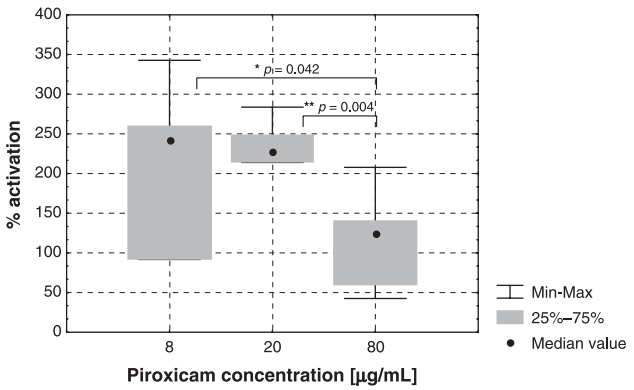

Figure 4. Effect of piroxicam on the maximum LCL intensity produced by a model system (HRP + H_2O_2 + luminol). Number of measurements: n (8 µg/mL) = 5; n (20 µg/mL) = 5; n (80 µg/mL) = 7.

MPO released, and is mediated by hypochloric acid (HOCl) formed in the MPO-catalysed-reaction. When phagocytes are stimulated by zymosan particles, a significant amount of MPO release occurs in parallel with a significant generation of superoxide. When phagocytes are stimulated by the chemotactic peptide fMLP, a substantial MPO secretion occurs, but the amount of superoxide produced is very small (30). Hence, in separate experiments, the effect of piroxicam on LCL by whole blood phagocytes in response to the chemoattractant fMLP was studied. We found that piroxicam markedly inhibited I_{max} of total LCL [median 68.9% (minimum value 46.1%, maximum value 75.9%)] and only slightly inhibited I_{max} of the simultaneously registered first phase of extra LCL [median 27.3% (minimum value 10.4%, maximum value 33.7%)] (Fig. 5). Therefore, the drug probably influences the process of degranulation or MPO-catalysed formation of HOCl. Such a supposition is in accordance with

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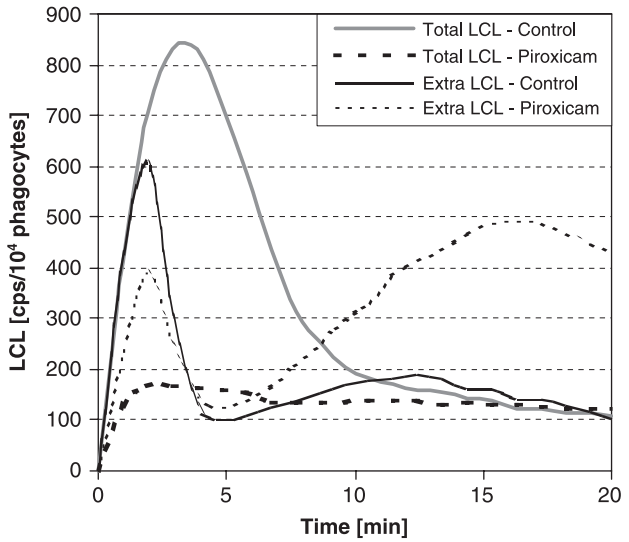

Figure 5. Effect of piroxicam (80 μg/ml) on fMLP-stimulated total and extra LCL; representative tracings of five experiments.

the study of Paino *et al.* (10), who have reported that piroxicam at therapeutic plasma concentrations scavenges pure HOCl and inhibits HOCl production in a model system containing MPO. Moreover, in cell systems, Shacter *et al.* (31) have also found that piroxicam inhibits the production of HOCl.

Piroxicam enhances zymosan-stimulated intracellular LCL (intra LCL)

Piroxicam slightly increased the intracellular light generation by phagocytes (defined as the activity that remained in a luminol-enhanced model system in the presence of SOD and CTS) at its lower concentrations ($H = 7.40$; $p = 0.02$) (Fig. 6). Such an effect is analogous to that observed in the total LCL at a lower stimulant concentration, which is considered predominantly

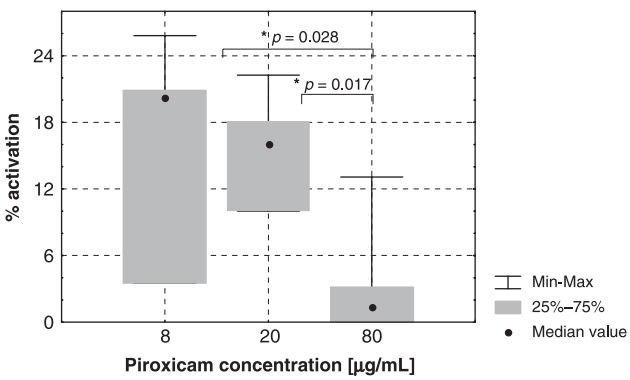

Figure 6. Effect of piroxicam on the maximum intensity of intra LCL (zymosan 4.0 mg/mL). Number of measurements: n (8 μg/mL) = 7; n (20 μg/mL) = 7; n (80 μg/mL) = 14.

intracellular (32). It is known that the inhibition of cyclooxygenase-1 by piroxicam leads to the accumulation of free arachidonic acid, which is able to directly activate the NADPH-oxidase through a protein kinase-independent pathway, which may explain the reversal of the effect. Furthermore, in the presence of piroxicam, more ROS may be produced via the 5-lipoxygenase pathway through leukotriene B4 (33).

Piroxicam effect is different on different sections of zymosan-stimulated LgCL kinetics

Data published regarding piroxicam effect on the extracellular superoxide production by lucigenin CL (LgCL) are controversial. Kapp *et al.* (34) determined that preincubation of granulocytes with piroxicam led to a reduced zymosan-stimulated lucigenin response only at high drug concentrations. On the other hand, Parij *et al.* (11) reported an increased LgCL by opsonized zymosan-stimulated isolated neutrophils. We found that piroxicam exerted a dose-dependent inhibitory effect on the initial section of the kinetics ($H = 7.64$; $p = 0.022$) and showed a tendency to enhance LgCL in later time intervals (Fig. 7A, B).

The results obtained with LgCL should be interpreted with caution, since under our experimental conditions the exogenous SOD did not completely eliminate the LgCL response (Fig. 7A). Such a result is in agreement with the observation that selective accumulation of the positively charged lucigenin molecule by mitochondria in cells may detect superoxide derived from mitochondrial respiration (35, 36).

CONCLUSION

We conclude that piroxicam dose-dependently inhibits zymosan-stimulated extracellular CL during the initial stages of phagocyte activation. Depending on the degree of stimulation, i.e. on the different relative contribution of the extracellular CL within the total response, the effect registered on totally generated light by phagocytes may vary from inhibitory to enhancing. Such a result may explain the controversial data cited regarding piroxicam effect on LCL by stimulated phagocytes. Piroxicam substantially reduces the integral fMLP-stimulated LCL and not so much the simultaneously registered extracellular LCL in the initial stages of phagocyte activation, i.e. it probably influences degranulation or MPO-catalysed generation of hypochloric acid. In the present study, we also demonstrate that piroxicam enhances CL in the presence of horseradish peroxidase, which could be observed at any concentration of the drug. Some additional data are needed to ascertain whether piroxicam changes HRP activity, i.e. increases peroxidase-catalysed decomposition of H_2O_2.

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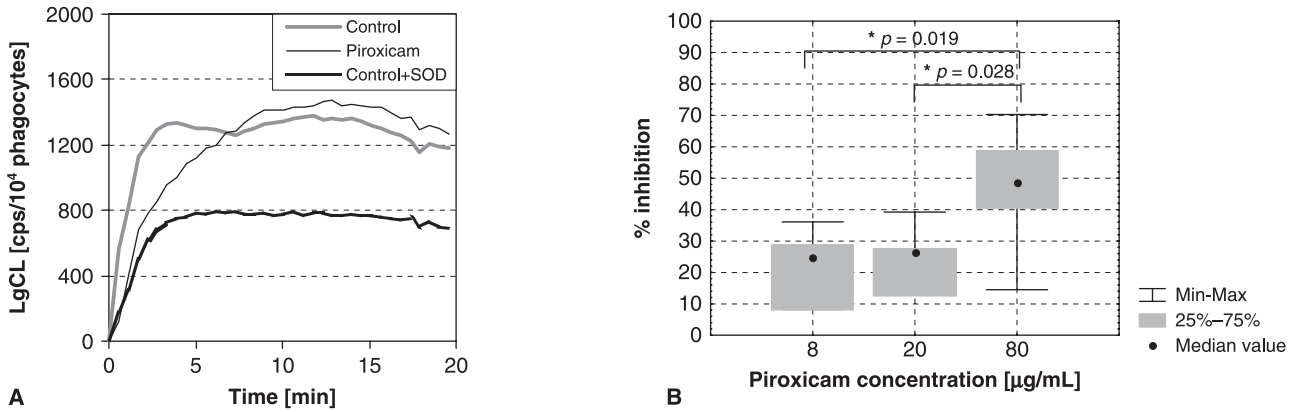

Figure 7. (A) Effect of piroxicam on LgCL (zymosan 4.0 mg/mL); representative tracings. SOD (150 U/mL) was added to the contents of the control system at zero time. (B) Dose-dependent inhibitory effect of piroxicam on LgCL intensity registered in the first minute after the start of response.

It is known that ROS are important mediators of cellular injury, playing a role in different pathological states. In this respect, the anti-inflammatory effect of piroxicam is related to inhibiting the extracellular generation of superoxide and hypochloric acid in the early stages of phagocyte activation.

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