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Lornoxicam Inhibits Human Polymorphonuclear Cell Migration Induced by fMLP, Interleukin-8 and Substance P

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Abstract: It has been established that nonsteroidal anti-inflammatory drugs (NSAIDs) have additional mechanisms of action, besides the inhibition of prostanoid production. We evaluated the ability of lornoxicam, a novel NSAID of the oxicam family, to interfere with the migration of human polymorphonuclear cells (PMN) *in vitro*. PMN were obtained from healthy donors. Chemotaxis was assessed by a Boyden microchemotaxis chamber and was stimulated with the bacterial chemoattractant formyl-met-leu-phe (fMLP, 0.1 μM), the chemokine Interleukin-8 (25 ng mL^{-1} , 0.003 μM) or the neuropeptide substance P (0.1 μM). Lornoxicam was used at the concentrations of 0.01, 1.0 and 100 μM . This NSAID inhibited fMLP and Substance P chemotaxis starting from the concentration of 1.0 μM , whereas Interleukin-8-induced chemotaxis was significantly reduced also at 0.01 μM . As these concentrations can be easily reached in plasma and in the synovial fluid after the administration of this drug *in vivo*, present data suggest that the inhibition of neutrophil migration is involved in the anti-inflammatory action of lornoxicam.

Key words: Lornoxicam, chemotaxis, fMLP, IL-8, SP

INTRODUCTION

Leukocytes are directed to sites of inflammation under the influence of a concentration gradient of soluble chemotactic molecules: this process is known as chemotaxis^[1].

Chemotaxis is therefore at the basis of the recruitment of inflammatory cells, such as polymorphonuclear cells (PMN) and monocytes, into a focus of inflammation or infection and of the following immune/inflammatory response. Chemotactic stimuli include both pathogen and host-derived substances^[2]. Among the pathogen derived factors an important role is played by the bacterial products formyl-met-leu-phe (fMLP) and lipopolysaccharide (LPS), while the host derived factors comprise the large family of chemokines as well as some neuropeptides such as Substance P^[2-4].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely and successfully used in the treatment of both acute and chronic inflammatory disorders. Although their activity is generally linked to the ability to inhibit prostanoid production, it is clear that this is not the only mechanism involved in their anti-inflammatory effects. In this context, the inhibition of the activity of inflammatory cells should be considered^[5,6]. In fact, NSAIDs belonging to different chemical classes have been shown to suppress a broad range of PMN neutrophil function^[3,5-10].

Lornoxicam is the latest proposed compound of NSAID oxicam class^[11]. It is a highly potent cyclooxygenase (COX) inhibitor, with a balanced activity on COX-1/COX-2^[11,12]. In the experimental animal, lornoxicam exhibited strong anti-inflammatory and anti-hyperalgesic activities^[13,14]. Its anti-inflammatory and analgesic properties were also demonstrated in the human^[11,15,16].

The bacterial product formyl-met-leu-phe (fMLP) is a classical and potent activating stimulus for PMN that induces chemotaxis, adherence and phagocytosis, therefore preparing cells for clearance of bacterial infections^[4,17].

Interleukin-8 (IL-8) is the prototype of the α (CXC) chemokines, a family of cytokines characterized by their ability to induce migration of PMN and that play a fundamental role in the beginning of the inflammatory response^[2]. Moreover, IL-8 is produced by activated synoviocytes, is present in the synovial fluid of arthritic patients and was suggested to have an important role in the pathogenesis of arthritic diseases^[18,19].

The undecapeptide Substance P (SP) is clearly involved in the modulation of the inflammatory response. It has been shown that it can promote migration of PMN and monocytes and induce synthesis of cytokines and prostanoids^[20,21]. High levels of SP are present in the synovial fluids of arthritic patients, where the peptide

contributes to the maintenance and development of inflammation and tissue destruction by attracting new inflammatory cells^[22-24].

For these reasons, this study investigate whether lornoxicam could interfere with the ability of human PMN to migrate in response to three different chemotaxis stimuli: fMLP, interleukin-8 (IL-8) and Substance P (SP).

MATERIALS AND METHODS

Human PMN preparation: Heparinized peripheral blood was obtained from healthy volunteers. Mononuclear cells were eliminated by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Milano, Italy) and remaining cells were sedimented on a dextran gradient for 30 min at 25°C in order to separate PMN. Hypotonic lysis of erythrocytes was accomplished by adding 0.2%NaCl for 20 sec, restoring isotonic saline with 1.6% NaCl and centrifuging the mixture at 300 g for 20 min. PMN (>98%) were diluted to the final concentration of 2×10^6 PMN mL⁻¹ in Dulbecco modified eagle medium (DMEM, Sigma St. Louis MO) to which 0.5% bovine serum albumin and 20 nM Hepes (Sigma St. Louis MO) were added, final pH 7.4 (chemotaxis buffer)^[25].

Substances: The chemotactic peptide formyl-Metionyl-Leucine-Phenylalanine (fMLP) (Sigma, St. Louis, MO) was stored as a stock solution of 10 mM in dimethyl sulphoxide (DMSO, Sigma, St. Louis, MO) at -80°C and diluted in chemotaxis buffer, (LPS free), just prior to the assay.

Substance P (Sigma, St. Louis MO) and human recombinant IL-8 (Tema Ricerca, Bologna, Italy) were prepared fresh in the chemotaxis buffer.

Lornoxicam (Prodotti Formenti, Milano, Italy) was prepared fresh for each experiment in the chemotaxis buffer.

Chemotaxis assay: Chemotaxis was measured using a Boyden modified 48-well microchemotaxis chamber, in which the upper and the lower compartments were separated by a polycarbonate filter (Biomap, Agrate Brianza, Italy), with a pore diameter of 3 µm. Cells (2×10^6 cells mL⁻¹, 1×10^5 PMN/ well) were placed in the upper chamber and DMEM alone or the chemoattractants were added in the lower chamber. The chambers were incubated for 45 min at 37°C, in an atmosphere of 5% CO₂ and then the migrated cells that adhered to the distal part of the filters were fixed and stained. Counting three fields

in triplicate with an optical image analyzer quantitated migrated cells^[3,25]. Results are expressed as chemotactic index, that is the ratio between the number of cells migrated toward test attractant and buffer alone.

Lornoxicam at concentrations of 0.01, 1 and 100 µM was added to the lower chemotaxis chamber without (in order to assess spontaneous migration), or together with 0.1 µM fMLP, 0.1 µM SP or 25 ng mL⁻¹ IL-8 (0.003 µM). The concentrations of chemoattractants were chosen on the basis of previous experiments, showing an optimal PMN chemotactic activity^[3,10,25]. Each experiment was repeated at least 5 times, using different donors. Statistical analysis of data was performed by ANOVA, followed by Bonferroni's t-test for multiple comparisons.

RESULTS

In the absence of chemotactic stimuli, spontaneous migration of PMN was 12.4 ± 3 (mean±SD) cells/microscopic field. Lornoxicam did not affect spontaneous migration of PMN (data not shown).

The addition of this NSAID significantly affected the chemotaxis stimulated by the different chemoattractant stimuli tested.

At the concentration of 0.1 µM fMLP induced a significant chemotaxis (chemotaxis index 2-3). As shown in Fig. 1, panel A, the addition of lornoxicam significantly decreased the ability of PMN to migrate toward fMLP. In the same panel the percentage of inhibition is reported: the maximal % inhibition obtained at 100 µM lornoxicam reached 70%.

As expected, IL-8 was a potent chemoattractant for PMN (Fig. 1, panel B). Lornoxicam reduced the chemokine-induced migration: a significant inhibition was already present at the low concentration of 0.01 µM, with a 30% inhibition that reached 70% at the two higher concentrations.

Lornoxicam reduced also SP-induced chemotaxis. As shown in panel C of Fig. 1, a statistical significant decrease (40%) of cell migration was observed at the concentration of 1 µM and of 58% at the concentration of 100 µM. A clear, although not statistically significant inhibition of 22% was already evident at 0.01 µM lornoxicam.

In order to rule out a direct toxic effect on PMN, at the end of the incubation period, the viability of cells was checked by trypan blue exclusion test. At none of the concentrations tested lornoxicam did affect the viability of PMN (% dead cells: medium alone 6.5 ± 2.78 ; lornoxicam 100 µM 7.5 ± 3.5).

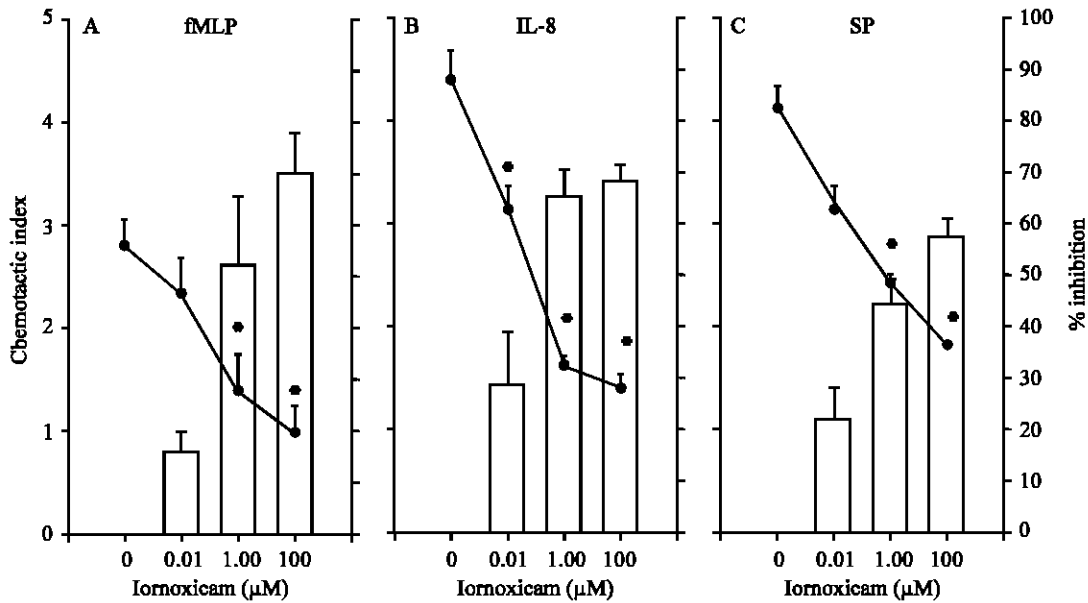


Fig. 1: Effect of lornoxicam at the concentration of 0.01, 1.0 and 100 μM on chemotaxis induced by 0.1 μM fMLP (panel A), 25 ng mL^{-1} IL-8 (panel B) and 0.1 μM SP (panel C). Results are expressed either as chemotactic index (left y-axis, lines) and as % inhibition of control (right y-axis, bars). Values are means \pm SD of five experiments. * $p < 0.05$ vs. migration without lornoxicam (0 drug, control)

DISCUSSION

It is widely accepted that the main mechanism of action of NSAIDs is the inhibition of prostanoid synthesis. However the inhibition of COX does not fully explain the anti-inflammatory activity of NSAIDs^[6]. Additional anti-inflammatory mechanisms include the inhibition of some leukocyte functions^[3,5,6,8].

The present study shows that lornoxicam, a novel NSAID of the oxamicam class, is able to interfere with the ability of PMN to respond to chemotactic stimuli. In fact, lornoxicam reduced the chemotaxis of PMN induced by fMLP, IL-8 and SP. The inhibition is achieved at a concentration of the drug as low as 0.01 μM when IL-8 was employed as stimulus. At 1 μM the migration response was inhibited up to 70%, depending on the stimulus used. In comparison with other drugs of the same pharmacological family, lornoxicam seems more potent in inhibiting chemotaxis; indeed piroxicam and tenoxicam have been demonstrated to interfere with PMN migration only starting from a concentration of 1 μM ^[7,10]. It is important to note that the concentrations required for lornoxicam inhibition of chemotaxis in our system can be achieved in plasma and in synovial fluid with the currently recommended doses in humans^[11]. The effects that we observed *in vitro* can therefore be relevant during anti-inflammatory therapies with this drug. Moreover the effects of lornoxicam were evident independently of the

activating stimulus utilized. Our choice of chemoattractant stimuli included the classical bacterial derived chemotactic peptide fMLP and two host-derived substances such as the chemokine IL-8 and the neuropeptide SP. IL-8 is a pivotal cytokine for PMN activation and for the onset and perpetration of the inflammatory reaction^[2,18,19]. The ability of lornoxicam to inhibit SP-induced chemotaxis might be particularly relevant for the treatment of arthritic diseases. In fact, high concentrations of SP are present in the synovial fluids of arthritic patients, where it could be delivered by free nerve terminals of the joints and by immune cells or both ways^[22,24]. Besides inducing chemotaxis, SP is a potent stimulator of prostaglandin and cytokine production^[21]. In the light of this fact, the reduction of SP-induced chemotaxis may contribute to interrupt the pathological loop of SP-induced stimulation of leukocytes to produce cytokines, that themselves induce pain and the release of more SP from nerve terminals^[20].

The mechanisms at the basis of PMN inhibition by NSAIDs have not been fully clarified^[6]. Ibuprofen has been shown to down regulate fMLP binding-affinity for its granulocyte surface receptor^[27]. However this is unlikely to be the specific effect of lornoxicam, since this drug inhibited also the chemotaxis induced by stimuli which bind to different receptors. It was also suggested that some NSAID could interfere with neutrophil G-protein^[28]. In particular, it has been demonstrated that

salicylate and piroxicam can disrupt the GTP/GDP exchange at the α subunit of G protein (28). As fMLP, IL-8 and SP receptors all belong to the G-protein receptor superfamily^[2,4,29], this mechanism cannot be excluded and deserves further investigation.

In conclusion, we demonstrated that lornoxicam is able to reduce the chemotaxis of human PMN. The direct effect exerted by lornoxicam on inflammatory cells can participate in its potent anti-inflammatory activity.

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