Inhibition of some human neutrophil functions by the cyclooxygenase inhibitor ketorolac tromethamine

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Abstract: Ketorolac tromethamine, a new nonsteroidal anti-inflammatory agent of the pyrrolo-pyrrrole group, was assayed for inhibitory effects on polymorphonuclear leukocytes (PMN) in a variety of systems. Ketorolac inhibited PMN superoxide anion generation, lysozyme release, myeloperoxidase release, adherence to plastic surfaces, and chemotaxis in response to N-formyl-methionylleucyl-phenylalanine (fMLP) in a dose-dependent manner. Ketorolac also inhibited phorbol myristate acetate-stimulated adherence of PMN to bovine pulmonary artery endothelial cells. The drug inhibited lysozyme and myeloperoxidase release by PMN in response to C5a but failed to inhibit C5a stimulation of PMN in any of the other assays. Levels of ketorolac required to inhibit PMN function in most systems were in the range of 0.2 to 1.0 mg/ml, but chemotaxis to fMLP was inhibited by concentrations of ketorolac as low as 1 µg/ml. Ketorolac, currently the only nonsteroidal anti-inflammatory drug available in a parenteral form may have therapeutic usefulness in a variety of conditions thought to be mediated in part by PMN, including sepsis. J. Leukoc. Biol. 51: 490-495; 1992.

Key Words: PMN • NSAID • inflammation

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAID) are a chemically diverse group of agents that suppress a broad range of inflammatory functions of polymorphonuclear neutrophils (PMN) by inhibition of cyclooxygenase [1, 5]. A large body of experimental data indicates that these drugs, especially ibuprofen, are useful in suppressing deleterious host responses to infection by gram-negative aerobic bacilli [3, 9, 16].

A new parenteral NSAID, ketorolac tromethamine, recently has been released principally for use as a non-narcotic analgesic. Kitorolac exhibits analgesic, anti-inflammatory, and antipyretic activities [12, 13]. It is a member of the pyrrolo-pyrrrole group of NSAIDs with a chemical name of (±)-5-benzoyl-2,3-dihydro-1H-pyrrrolizin-1-carboxylic acid, 2-amino-2-(hydroxymethyl)-1,3-propanediol. The anti-inflammatory activity of this class of agents seems most pronounced in models of acute injury. For instance, in one series of studies, the drug was much more effective in reducing carrageen-induced rat paw edema than it was in inhibiting chronic cotton pellet granuloma or adjuvant-induced arthritis [4, 15].

Because the drug is the only NSAID currently available for parenteral injection, we tested its inhibitory effects on neutrophil functions in some systems previously studied with ibuprofen [10]. We hypothesized that this parenteral cyclooxygenase inhibitor might have useful anti-inflammatory properties in models of bacterial sepsis similar to those seen with ibuprofen [3, 9, 16].

METHODS

Neutrophil Preparation

Human neutrophils were obtained by a modification of the method of Boyum [4] using dextran sedimentation and gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) followed by hypotonic lysis of red blood cells. Resulting preparations of neutrophils were more than 98% pure and 98% viable by trypan blue exclusion. Subjects were healthy volunteers who had been medication-free for 7 days and had given informed consent.

Preparation of Reagents

C5a isolated from zymosan-activated human serum [11] was a generous gift from Dr. Robert O. Webster. Ketorolac tromethamine (Toradol, Syntex Laboratories, Palo Alto, CA) at a concentration of 30 mg/ml in 10% ethyl alcohol was obtained from Hoffmann-La Roche (Nutley, NJ). In experiments where this form of ketorolac was used, a control of 10% ethyl alcohol in normal saline at the smallest dilution of ketorolac used (1:30) was included. In addition, ketorolac tromethamine with no ethanol (generously provided by Syntex Laboratories) was tested once in every series of experiments to assure that its effect remained comparable in the absence of ethyl alcohol. Ibuprofen (50 mg/ml in glycerine/saline buffer, a generous gift of The Upjohn Company, Kalamazoo, MI) was tested at 1 mg/ml in some experiments as a positive control for inhibition of PMN functions.

Granule-Associated Enzyme Release

Neutrophil myeloperoxidase (MPO) and lysozyme were measured by the method of Henson [7, 8] in a 1.0-ml vol containing 4 × 10⁶ neutrophils preincubated with 5 µg/ml cytochalasin B (Sigma Chemical Co., St. Louis, MO) for 5 min at 37°C. Various concentrations of ketorolac were diluted in Hanks' balanced salt solution containing 0.25% bovine serum albumin (HBSS-BSA) (both from Gibco Laboratories, Grand Island, NY). Neutrophils were preincubated with these dilutions or with HBSS-BSA for 5 min at 37°C. C5a was added to a final concentration of 2 nM or N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma) was added to a final concentration of 50 nM and incubated
for 15 min at 37°C. Reaction mixtures then were centrifuged (800g, 5 min) at 4°C, and cell-free supernatants were removed for enzyme assays. Total enzyme activity was assessed by lysing an identical reaction mixture with Triton x-100 (0.1% v/v; Sigma). Background of cells with no stimulus and no inhibitor was subtracted from each value before obtaining percent of total release and percent inhibition of control (fMLP- or C5a-stimulated cells with no inhibitor).

Lactate dehydrogenase (LDH) was measured on these same supernatants to determine whether ketorolac was causing cell injury. LDH-Statzyme (Technicon Diagnostic System, Tarrytown, NY) spectrophotometrically measured the LDH-catalyzed conversion of lactate + NAD to pyruvate + NADH. The increase in absorbance of NADH is measured at 340 nm after addition of 0.1 ml of cell supernatant to 3 ml of LDH reagent.

**Superoxide Anion Generation**

Generation of superoxide anion radicals by neutrophils was measured as superoxide dismutase-inhibitable reduction of ferricytochrome C [11]. Neutrophils were preincubated with 5 µg/ml cytochalasin B for 5 min at 37°C then added to various dilutions of ketorolac or HBSS-BSA for a 10-min incubation at 37°C. Final reaction mixtures contained pretreated neutrophils (2.5 x 10⁶ per ml), 0.08 mM cytochrome c (Sigma), and either C5a (10 nM), fMLP (50 nM), or HBSS-BSA for background control. After a 15-min incubation at 37°C, reaction mixtures were centrifuged (500g, 10 min) at 4°C, and supernatants were decanted and read at 550 nm. Dilutions of ketorolac were tested for oxygen radical scavenging in a cell-free system measuring cytochrome c reduction mediated by the aerobic action of xanthine oxidase on hypoxanthine [6]. Superoxide anion generation is expressed as nanomoles of cytochrome c reduced per reaction mixture per 15 min.

Some superoxide anion generation experiments were performed with ketorolac addition after fMLP stimulation to determine whether pretreatment with ketorolac was necessary to see an inhibitory effect. In these experiments, reaction mixtures containing fMLP (or HBSS-BSA for background) and cytochrome c were incubated for various periods of time before addition of ketorolac (or addition of HBSS-BSA in control tubes). The reaction then was allowed to proceed for an additional 10 min at 37°C before centrifugation and reading as above.

To determine whether inhibition of superoxide anion by ketorolac required the continuous presence of the drug, washing experiments were performed. Neutrophils were pretreated (with or without ketorolac) for 10 min at 37°C as above then either treated with cytochalasin B and added to the reaction mixture containing stimulus and cytochrome c as above or washed first (one to three times). Washes were done by dilution of neutrophils pretreated with or without ketorolac to 10 ml with calcium- and magnesium-free phosphate-buffered saline (PBS), pH 7.2, and centrifugation (150g, 10 min) at 4°C. Washed cells were resuspended to 2.5 x 10⁶ per ml in HBSS-BSA and incubated in the reaction mixture as above.

**Chemotaxis**

Neutrophil chemotaxis was measured in a 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD) using a 3.0-µm cellulose nitrate filter (Neuroprobe). Stimulus (fMLP at 10 nM or C5a at 1 nM) or buffer (HBSS with 1% BSA and 10 mM HEPES) was placed in the lower wells and covered with the filter. Cells (3.5 x 10⁶ per ml) that had been preincubated 10 min with various concentrations of ketorolac or HBSS-BSA were added to the top wells and incubated at 37°C for 35 min. Filters were removed, fixed in 2-propanol, and stained with Harris hematoxylin. After clarification in xylene, filters were mounted on microscope slides and read by the leading-front method [19]. Five 400x fields were measured on each well, and all samples were assayed in duplicate. Random migration (microns moved in response to HBSS-BSA alone) was subtracted from each mean, and values were calculated as net migration. Chemotaxis assays were performed with alcohol-free ketorolac.

Chemokinesis assays were performed by placing an equal concentration of stimulus above and below the filter at various concentrations in the presence of PMN pretreated with ketorolac or buffer alone to determine the effect of ketorolac on fMLP-stimulated neutrophil chemokinesis.

**Neutrophil Adherence**

Adherence assays to plastic surfaces or endothelial cell monolayers were performed by the method of Webster [18] in 24-well tissue culture plates (Corning Glass, Oneonta, NY). For endothelial cell experiments, bovine pulmonary artery endothelial cells (American Type Culture Collection, Rockville, MD) were grown to confluence in plates with Eagle's MEM with 10% heat-inactivated fetal bovine serum (MEM-FBS, JRH Biosciences, Lenexa, KS) and used at 3 days postconfluence. Alcohol-free ketorolac was used for adherence experiments. Dilutions of ketorolac were made in MEM-FBS and added to neutrophils (final concentration 2 x 10⁶ per ml). MEM-FBS alone was added to an identical neutrophil mixture as a control, and all neutrophils were incubated in triplicate in wells with and without stimulus (fMLP, 1 µM, C5a, 50 nM; or phorbol myristate acetate [Sigma], 2.5 ng/ml) for 30 min at 37°C under 5% CO₂. Supernatants then were decanted, and plates were washed twice with Dulbecco's PBS containing calcium and magnesium. Neutrophils remaining adherent to the plate were lysed with Triton x-100 (0.2% v/v), and the supernatants were assayed for MPO activity as described above. Total MPO activity was assessed by lysis of 2 x 10⁶ neutrophils, and inhibition was calculated from percent of total enzyme released.

**Leukotriene Assay**

The possible modulation of fMLP-stimulated leukotriene B₄ (LTB₄) production in PMN by ketorolac was assayed in supernatants from one of the degranulation experiments described above. After preincubation with ketorolac and stimulation with fMLP, generation of leukotrienes was stopped by addition of 200 µg/ml of the inhibitor BW755C (Wellcome Research Laboratories, Kent, UK). Cells were removed by centrifugation (800g, 5 min) at 4°C. One ml of supernatant was decanted and added to 4 ml of ice-cold methanol and frozen at -20°C overnight. This mixture then was centrifuged (800g, 10 min) at 4°C, and the supernatant was dried down in a speed-vac centrifuge and assayed by enzyme immunoassay for LTB₄ [13].

**RESULTS**

Ketorolac inhibited neutrophil adherence, degranulation, and superoxide anion release in a dose-dependent fashion. The effects were seen at drug concentrations varying from less than 200 µg/ml up to 1 mg/ml. Viability of cells in-
cubated with these doses of ketorolac for 10 min at 37°C was greater than 98% by trypan blue exclusion. LDH-release experiments also indicated that cells were viable after incubation with ketorolac. In three separate experiments done in duplicate, all doses of ketorolac tested (0.01 mg/ml to 1 mg/ml) resulted in mean changes in absorbance per minute with LDH reagent equal to or less than those with cell blank supernatant (mean ΔA₄₁₀ = 0.0031 ± 0.0004), while total tube supernatants produced mean changes in absorbance per minute of 0.0539 ± 0.004.

Neutrophil adherence to plastic surfaces was first tested in the presence or absence of ketorolac using fMLP (1 μM) or C5a (50 nM) as the stimulus for adherence. Ketorolac inhibited fMLP-stimulated adherence in a dose-dependent fashion from 200 μg/ml to 1 mg/ml but did not inhibit C5a-stimulated adherence (Fig. 1). Ibuprofen preincubation at 1 mg/ml inhibited 1 μM fMLP-stimulated adherence to plastic by 62%. Ketorolac inhibited PMA-induced stimulation of neutrophil adherence to cultured bovine pulmonary artery endothelial cells (Fig. 2). The effect seemed linearly related to drug concentration between 0.4 mg/ml and 1.0 mg/ml.

Neutrophil degranulation and release of MPO and lysozyme was tested using 50 nM fMLP (Fig. 3) or 2 nM C5a (Fig. 4) as stimulus. Ketorolac inhibited release of both granular enzymes released by both stimuli in a dose-dependent fashion between the tested concentrations of 50 μg/ml and 1 mg/ml. Inhibition of MPO release by fMLP-stimulated neutrophils approached 80% at ketorolac concentrations of 1 mg/ml. With 50 nM fMLP as stimulus, pretreatment of PMN with 1 mg/ml of ibuprofen caused 98% inhibition of MPO release and 77% inhibition of lysozyme release.

The effect of ketorolac on neutrophil generation of superoxide anion was tested using fMLP (50 nM) or C5a (10 nM) as stimulus. Ketorolac suppressed superoxide anion generation by fMLP in a dose-dependent manner over the concentration range tested (Fig. 5) but did not inhibit C5a-stimulated superoxide anion generation (data not shown). Pretreatment of PMN with 1 mg/ml of ibuprofen inhibited superoxide anion generation by 50 nM fMLP by 99%. Ketorolac added to the reaction mixture up to 10 min after fMLP stimulation could stop the accumulation of superoxide anion to some extent (Fig. 6). However, by 10 min after the addition of fMLP, the reaction had been completed; the addition of ketorolac no longer had any effect. Ketorolac showed no ability to scavenge superoxide anion in a xanthine oxidase–hypoxanthine system (data not shown).

The continuous presence of ketorolac seemed to be required for inhibition of superoxide anion generation by fMLP (Fig. 7). Each wash of ketorolac-treated neutrophils before stimulation increased the amount of superoxide anion generated after stimulation with fMLP; in neutrophils pretreated with buffer only, however, a decrease in superoxide anion production was observed after stimulation. Myeloperoxidase assays of neutrophils after each wash showed no loss of neutrophils from any wash in terms of absolute numbers (data not shown).
Fig. 4. C5a-stimulated release of granule-associated enzymes from neutrophils also was inhibited by ketorolac. Mean percent inhibition on MPO (O) and lysozyme (A) ± SEM was determined in three separate experiments, each performed in duplicate. Release of enzyme in the absence of inhibitor by 2 nM C5a was 18 ± 2 and 39 ± 3% of total enzyme for MPO and lysozyme, respectively. Ethanol-containing vehicle control inhibited C5a-stimulated MPO (O) and lysozyme (A) by 25 ± 8 and 18 ± 6%, respectively.

The effect of ketorolac on chemotaxis of neutrophils to a concentration gradient of fMLP (10 nM) in four experiments was a modest inhibition over the range from 1 μg/ml to 1 mg/ml (Fig. 8), while pretreatment with 1 mg/ml of ibuprofen inhibited chemotaxis by 100% at this dose of fMLP. At 50 nM fMLP in five experiments, ketorolac inhibited neutrophil chemotaxis by an average of 20–30% at concentrations from 200 μg/ml to 1 μg/ml, but ketorolac at 1 mg/ml actually enhanced chemotaxis by an average of 14% (data not shown). Ketorolac did not inhibit C5a-stimulated chemotaxis at any dose tested (1 μg/ml to 1 mg/ml).

Assays to determine the effect of ketorolac on fMLP-stimulated PMN chemokinesis showed a slight inhibition of chemokinesis at high concentrations of ketorolac and low concentrations of fMLP (0.5 nM), but ketorolac seemed to increase chemokinetic activity at high concentrations of fMLP (10 nM) (Table).

Ketorolac inhibited fMLP-stimulated LTB4 production in PMN supernatants by 52% at 1 mg/ml, 39% at 0.1 mg/ml, and 32% at 0.01 mg/ml. fMLP-stimulated supernatants alone contained 137 pg/ml LTB4.

Ketorolac is commercially available dissolved in a carrier containing ethyl alcohol 10% (vol/vol). Because alcohol also can suppress neutrophil function, we performed selected experiments with an alcohol-free ketorolac preparation or with ethyl alcohol in saline in the appropriate dilution as a control. In general, the alcohol-free ketorolac performed similarly to the commercially available product. Results of these control experiments are shown in the figures for the experiments in which ketorolac with 10% ethanol vehicle was used.

Fig. 6. Superoxide anion produced by neutrophils stimulated by 50 nM fMLP with (O) or without (□) ketorolac (0.5 mg/ml) added at intervals after stimulation is expressed as mean (± SEM) nM of cytochrome c reduced per 2 million neutrophils per 10 min. The x-axis represents minutes after stimulation with fMLP before ketorolac was added. All reactions proceeded for 10 min past the time of addition of ketorolac, and each time point had its own control tubes, with no ketorolac incubated for an identical length of time.

Fig. 5. Ketorolac inhibition of superoxide anion generation by 50 nM fMLP was assessed after pretreatment of neutrophils with and without drug for 10 min at 37°C. Ketorolac (O) inhibition and inhibition by ethanol-containing vehicle (□) are expressed as mean percent inhibition ± SEM in four separate experiments, each performed in duplicate. Stimulation by fMLP with no inhibitor produced 63 ± 10 nM cytochrome c reduced per 2 million neutrophils per 15 min.

Fig. 7. Superoxide anion production stimulated by 50 nM fMLP in neutrophils pretreated for 10 min at 37°C with (O) or without (□) ketorolac (1 mg/ml) is expressed as mean nanometers ± SEM of cytochrome c reduced per 2 million neutrophils per 15 min. Some neutrophils were washed in PBS up to three times before stimulation with fMLP.
Ketorolac in these experiments behaved like other NSAID, which exhibit stimulus-specific inhibition of neutrophil inflammatory functions [1, 11]. In addition to blockade of the cyclooxygenase pathway, ketorolac may act directly on neutrophils through one or more of the other mechanisms suggested for NSAID. Ibuprofen down-regulates fMLP-binding affinity to its granulocyte surface receptor, for example [17]. Another suggested mechanism of NSAID inhibition of PMN function is blockade of pertussis toxin–dependent ADP-ribosylation of the G protein of the neutrophil [2].

Since cyclooxygenase products are not the major products of arachidonic acid in PMN, LTB4 was measured in our fMLP-stimulated PMN system with and without ketorolac. Ketorolac was found to have some inhibitory effect on fMLP-stimulated LTB4 production in isolated PMN, which could be one mechanism by which ketorolac exerts an anti-inflammatory effect. However, leukotriene production by PMN at the dose of fMLP used in our degranulation assay was in the picomolar range, somewhat less than the concentration usually required to promote such PMN functions as chemotaxis [10].

The observed increase in PMN chemokinesis at optimal doses of fMLP and high doses of ketorolac is similar to the findings of Perianin et al. [12] with indomethacin. However, indomethacin only increased chemokinesis at a chemokinetically inactivating dose of fMLP (100 nM), while ketorolac increased the already enhanced chemokinesis of PMN at 10 nM fMLP, indicating that different mechanisms may be involved. Similarly, at concentrations of fMLP higher than the optimum chemotactic concentration, 1 mg/ml ketorolac actually seemed to enhance chemokinesis, possibly because the drug interfered with the interaction of fMLP with its receptor.

Ketorolac resembled ibuprofen in its inhibitory effects on some neutrophil functions in vitro [11]. The drug's effects on superoxide anion release and degranulation exhibited a dose-dependent inhibition, with maximum effects seen at concentrations not usually achieved pharmacologically. Many NSAID act as antipyretics and analgesics at much lower doses than required for anti-inflammatory action [2]. However, the effect on directed neutrophil migration (chemotaxis) was evident at much lower concentrations than the effects on superoxide anion generation or degranulation. The levels required for inhibition of chemotaxis in our system easily could be achieved in vivo with currently recommended doses. Since neutrophil influx into the lungs and other organs is one of the early injurious events in sepsis, inhibition of neutrophil chemotaxis could be an important protective factor in this illness. Further studies will be necessary to determine whether or not ketorolac has the same protective effect as ibuprofen when used in animal models of sepsis.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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**TABLE 1. Effect of Ketorolac Pretreatment on fMLP-Stimulated Neutrophil Chemokinesis**

<table>
<thead>
<tr>
<th>Concentration of ketorolac (mg/ml)</th>
<th>Concentration of fMLP (ng/ml)</th>
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<tbody>
<tr>
<td>None</td>
<td>0.1</td>
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<tr>
<td>0</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>0.5</td>
<td>62 ± 4</td>
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<tr>
<td>1.0</td>
<td>67 ± 6</td>
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<td>5.0</td>
<td>73 ± 3</td>
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<tr>
<td>10.0</td>
<td>68 ± 5</td>
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*Neutrophils were pretreated with buffer or ketorolac for 5 min at 37°C before addition of fMLP.

†Results represent mean ± SEM of 10 high power fields on duplicate filters and are representative of four experiments.

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**Fig. 8.** Chemotaxis of PMN to 10 nM fMLP was measured in a microchemotaxis chamber for 35 min after pretreatment for 10 min at 37°C with various concentrations of ketorolac. Data are expressed as mean percent inhibition ± SEM in four experiments.


