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Selective inhibition of interleukin-8-induced neutrophil chemotaxis by ketoprofen isomers

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Abstract

Although it is commonly accepted that the anti-inflammatory effect of nonsteroidal anti-inflammatory drugs (NSAIDs) is mainly associated to their ability to inhibit the cyclooxygenase (COX) enzyme system, several results indicate that non-COX mechanisms could be important in the therapeutical effect of these drugs. The aim of this study was to define if NSAIDs could exert, at least in part, their anti-inflammatory effect by inhibiting the activities of human polymorphonuclear leukocytes (PMNs) triggered by chemotactic stimuli and, if so, to understand the relationship of this effect with COX inhibition. A unique opportunity to dissociate the inhibition of prostaglandin (PG) synthesis from other therapeutical properties of NSAIDs is constituted by ketoprofen isomers being the S-isomer 100 time more potent than R-isomer on COX inhibition. Our results show that R- and S-ketoprofen, independently of their potency as PG inhibitors, proved very efficacious in selective inhibition of interleukin-8 (IL-8) chemotaxis. Inhibition of IL-8 chemotaxis was not restricted to ketoprofen isomer as it could be observed also with drugs belonging to different classes of NSAIDs and it was obtained at drug concentration superimposable to plasma levels after therapeutic administration in patients. Reduction of IL-8 migration by ketoprofen isomers was paralleled by selective inhibition of PMN response in terms of intracellular calcium concentration ($[Ca^{2+}]_i$) increase and extracellular signal regulated kinase (ERK)-2 activation, two intracellular mediators reported to be critical for PMN activities. It is concluded that inhibition of IL-8 chemotaxis could represent a new clinical target for ketoprofen isomers and, in fact, contribute to the anti-inflammatory activity of NSAIDs.

Keywords: Interleukin-8; Neutrophils; Chemotaxis; Ketoprofen; Isomers; Intracellular mediators

1. Introduction

It is generally accepted that the therapeutic effect of NSAIDs is mainly associated to their ability to inhibit the COX enzymes, thereby blocking the synthesis of PGs. Nevertheless, different recent reports suggest that non-PG mechanisms could be also important in the anti-inflammatory and analgesic action NSAIDs [1,2]. Among these mechanisms it was hypothesized that inhibition of PMN activation by NSAIDs could be a major PG-independent target [2–7]. PMNs play a critical role in the pathogenesis of inflammation, septic shock and acute-phase response. PMNs respond to a variety of inflammatory mediators by marginating to endothelium and by migrating from the blood stream to the site of inflammation [8,9]. Thus blocking these cells could be of importance in NSAID activity. However, the concentrations of NSAIDs reported to inhibit

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Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase; PG, prostaglandin; PMN, human polymorphonuclear leukocyte; IL-8, interleukin-8; $[Ca^{2+}]_i$, intracellular calcium concentration; MAPK, mitogen-activated protein kinases; fMLP, N-formyl-methionyl-leucyl-phenylalanine; ERK, extracellular signal regulated kinase; C5a, fifth component of complement; MCP-1, monocyte chemotactic protein-1; NAP-2, neutrophil activating protein-2; GCP-2, granulocyte chemotactic peptide-2; PVP, polyvinylpyrrolidone; HBSS, Hank's balanced salt solution; ENA-78, epithelial-derived neutrophil activating protein-78; ECL, enhanced chemiluminescent; FURA-2AM; 1-[2-(5-carboxyoxazol-2-yl)-6-amino-benzofuran-5-oxy]-2-(2′-amino-5′-ethlphenoxy)-ethane-N,N,N′,N′-tetraacetic acid pentaacetoxy methylester; MGSA/GRO, melanoma growth stimulatory activity/gene product of *gro* gene.

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PMN activation are well higher than those achievable in plasma exhibiting an anti-inflammatory effect [4–6]. A possible explanation of this discordance is the fact that the inhibitory effect of NSAIDs on PMN activities was observed against bacterial (e.g. fMLP) or non physiologic stimuli (e.g. phorbol myristate acetate and calcimycin), therefore their activity could be underestimated.

IL-8 is a major mediator of PMN recruitment in several inflammatory diseases including psoriasis [10], rheumatoid arthritis [11], ulcerative colitis [12], idiopathic pulmonary fibrosis and adult respiratory distress syndrome [13,14]. IL-8 is a protein that belongs to the C-X-C branch of chemokine family, which includes also MGSA/GRO, NAP-2, ENA-78, and GCP-2. IL-8 receptors belong to the seven-transmembrane-domain family and they have been shown to couple to an intracellular signal transduction pathways involving the activation of *Bordetella pertussis* toxin-sensitive GTP-binding proteins, activation of phopholipase C, formation of the second messenger inositol 1,4,5-trisphosphate and the subsequent increase in $[Ca^{2+}]_i$ [9,15]. Levels of Ras GTP have been shown to increase in response to IL-8 and activity of the downstream MAPK, also known as ERK, became stimulated by IL-8 [16].

The aim of this study was to define if NSAIDs could exert, at least in part, their antiinflammatory effect by inhibiting the activities of PMNs triggered by IL-8, and, if so, to understand the relationship of this effect with COX inhibition. A unique opportunity to dissociate the inhibition of PG synthesis from other therapeutic properties of NSAIDs is constituted by isomers of 2-arylpropionic acids. The 2-arylpropionic acids are a well-known class of NSAIDs that includes ketoprofen, flurbiprofen and ibuprofen. Due to the presence of an asymmetric carbon atom in the α-position to the carbonyl function, these drugs are chiral compounds and are generally used as a racemic mixture of two enantiomeric forms: R and S-isomers. Although the anti-inflammatory role of the two enantiomers is not fully characterized, it is known that the S-isomers are 100-1000 times more potent than R-isomers on COX inhibition [17–19]. R-isomers of 2-arylpropionic acids are known to transform to the S-isomers in vivo in several species [1,19,20]. However, the inversion of R-ketoprofen has been found in dogs and rats while no interconversion were observed in humans [1]. Inversion of R-isomers occurs mainly, if not exclusively, in liver tissue, while no interconversion was reported in vitro in leukocytes [4,21].

The results reported hereafter show that R and S-ketoprofen drammatically and selectively inhibited IL-8-induced PMN chemotaxis while a slight inhibition was observed on PMN degranulation. R and S-ketoprofen, in a therapeutic range of concentrations, equally reduced IL-8 activities. The inhibitory effect of ketoprofen enantiomers was paralleled by a significant decrease of PMN response to IL-8 in terms of $[Ca^{2+}]_i$ and ERK activation.

2. Materials and methods

2.1. Reagents and chemicals

Recombinant IL-8, MGSA/GROα and MCP-1, were purchased from PeproTech. [^{125}I]IL-8 (specific activity 2200 Ci/mmol) was from Du-Pont-NEN. fMLP and C5a were from Sigma Chemical Co. Ficoll/Hipaque, Percoll and dextran were from Pharmacia LKB. PBS, BSA, Cytochalasin B, FURA-2AM were from Sigma. HBSS was from Irvine. RPMI 1640 was from Gibco. HEPES, Triton X-100, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, aprotinin, sodium orthovanadate, NaF, β-glycerophosphate, sodium pirophosphate, β-mercaptoethanol were from Sigma. Diff-Quik was from Harleco. Micro Boyden chambers and polycarbonate filter were from Neuroprobe Inc.

Ketoprofen, ibuprofen and flurbiprofen isomers and racemate were from Dompé S.p.A. The R and S- ketoprofen isomers were dissolved using stoichiometric amounts of 50% DL-lysine water solution and diluted to appropriate concentrations in saline. Enantiomers of flurbiprofen and ibuprofen were solubilized using an equimolar NaOH solution and diluted to appropriate concentrations in saline. Ketoprofen, flurbiprofen and ibuprofen racemates were a mixture of equal amounts of R- and S-isomers (50% of each isomer). Indomethacin and diclofenac were from Sigma and were solubilized in DMSO and diluted to appropriate concentrations in saline.

2.2. Cells

Human mononuclear cells and PMNs were obtained from buffy coats of heparinized blood from normal volunteers through the courtesy of Centro Trasfusionale, Ospedale S. Salvatore, L'Aquila, Italy. Mononuclear cells were obtained by centrifugation on Ficoll/Hipaque. Monocytes were separated by Percoll gradient centrifugation [22]. Human PMNs were prepared to 95% purity by dextran sedimentation followed by hypotonic lysis of contaminating red blood cells [23]. Cellular viability was >95% in all experiments, as measured by trypan blue dye exclusion.

2.3. Migration assay

Cell migration for human monocytes and PMNs was evaluated using a 48-well micro-chemotaxis chamber, as previously described [24]. Twenty-five microliters of control medium (PBS for monocytes and HBSS for PMN, with 0.2% BSA) or chemoattractant solution were seeded in the lower compartment of the chemotaxis chamber. Fifty microliters of cells suspension (1.5×10^6/mL) preincubated at 37° for 15 min in the presence or absence of different concentrations of ketoprofen isomers were seeded in the upper compartment. Control samples received DL-lysine/saline solution at the appropriate dilution (vehicle). The two compartments of the chemotactic chamber were separated

by a 5-μm polycarbonate filter (PVP-free for PMN chemotaxis). The chamber was incubated at 37° in air with 5% CO_2 for 45 min (PMNs) or for 2 hr (monocytes). At the end of incubation, filters were removed, fixed, stained with Diff-Quik and five oil immersion fields at high magnification (100×) were counted after sample coding.

2.4. Intracellular calcium measurement

Adherent PMNs on coverslips were loaded with FURA-2AM, washed, and incubated at 37° with the different stimuli. Fluorescence was monitored using an epifluorescence microscope equipped with fluorescence optics and dichroic mirror appropriate for FURA-2 fluorescence. FURA-2 was excited at 350 and 380 nm every second and the emitted fluorescence was filtered between 510 and 530 nm and monitored using a CCD camera (Dage MTI) and a Georgia Instruments Image Analyzer. Regions of interest corresponding to individual cells were identified in each experiment, and average fluorescence was recorded and stored as individual data files. Fluorescence intensity was converted into $[Ca^{2+}]_i$ as previously described [25]. Representative experiments are shown as fluorescence tracings of individual cells. Results from several experiments are also summarized as number of responsive cells. Cells were considered responsive when the stimulus-induced increase of $[Ca^{2+}]_i$ was more than 30% over the baseline (normalized to 100%).

2.5. Measurement of elastase release

Isolated PMNs were resuspended (10^7/mL) in PBS containing 0.1% BSA and preincubated with R or S-ketoprofen or vehicle for 15 min at 37°. After 5 min of preincubation, cytochalasin B 10^{-5} M was added to the samples. Then cells, divided in aliquotes of 200 μl, were stimulated with 200 μL of IL-8 (50 ng/mL) diluted in PBS, 0.1% BSA, supplemented with $CaCl_2$ and $MgCl_2$ to yield a final concentration of 0.9 and 0.5 mM, respectively. After 30 min, samples were centrifuged and supernatants analyzed for elastase activity. Elastase enzymatic activity in PMN supernatants was measured at 410 nm as hydrolysis of MeO-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (600 μM final concentration). Results are expressed as variations of optical density per minute (Δ OD $\times$ 10^3/min) recorded in the first 5 min of the assay. During this time enzymatic kinetics are linear [26].

2.6. Western blot analysis of MAP kinases

Total PMNs (5×10^6 cells/mL) were prepared using a lysis buffer (20 mM Tris-HCl pH 7.4; 2 mM EDTA; 1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride; 0,1 mM leupeptin; 0.05 mM pepstatin A; 2000 U/ml aprotinin; 0.2 mM sodium orthovanadate; 25 mM NaF; 25 mM β-glycerophosphate; 25 mM sodium pyrophosphate and 5 mM β-mercaptoethanol). Proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose (Biobond-NC 0.2 μm, Whatman), and the quality of the transfer was evaluated with Ponceau staining. Nitrocellulose was blocked with non fat dry milk and then incubated for 1 hr at room temperature with Phospho-specific anti-active MAPK polyclonal antibody (Promega Corporation) or anti MAPK-R2 antibody (Upstate Biotechnology Corporation). All antibodies were used at 1:1000 dilution. Immunoreactivity was evaluated with anti-rabbit horseradish peroxidase antibody (Amersham Pharmacia Biotech), and by ECL detection system (Amersham).

2.7. IL-8 binding assay

Isolated PMNs ($10^7 \times$ mL) were resuspended in RPMI 1640 and incubated at 37° for 15 min in the presence of ketoprofen isomers (1 μM) or vehicle. After incubation cells were resuspended (2×10^7/mL) in binding medium (RPMI 1640 containing 10 mg/ml BSA, 20 mM HEPES, and 0.02% NaN_3) in the presence of ketoprofen isomers or vehicle. Aliquots of 0.2 nM of [^{125}I]IL-8 and serial dilutions of unlabeled IL-8 were added to 10^6 cells in 100 μL of binding medium and incubated at room temperature for 1 hr under gentle agitation. Unbound radioactivity was separated from cell-bound radioactivity by centrifugation through an oil gradient (80% silicon and 20% paraffin) on a microcentrifuge. Non-specific binding was determined by adding a 100-fold molar excess of unlabeled IL-8. Scatchard analysis and all calculations were performed with the LIGAND program [27].

3. Results

3.1. Inhibition of IL-8-induced PMN activities

The effect of ketoprofen enantiomers on IL-8-induced PMN activities was assessed in a therapeutic (0.1–1 μM) [28] as well as in a lower concentration range (0.1–10 nM). As reported in Fig. 1, pretreatment of PMNs with 1 nM R-ketoprofen (Fig. 1A) or S-ketoprofen (Fig. 1B) significantly reduced PMN migration induced by an optimal concentration (10 ng/mL) [29] of IL-8. Ketoprofen isomers were equally efficacious in inhibiting IL-8 chemotaxis. Reduction of PMN migration by R and S-ketoprofen was concentration dependent, being almost complete at 1 μM (96% and 89% of inhibition by R and S-ketoprofen, respectively). A comparable inhibition of IL-8 chemotaxis by R and S-ketoprofen was also observed in the presence of serum (HBSS with 1% fetal calf serum as assay medium; data not shown).

Under the same experimental conditions, R- and S-ketoprofen also inhibited PMN migration induced by MGSA/GROα (100 ng/mL), a chemokine belonging to the same C-X-C family. As shown in Fig. 2, R and S-ketoprofen (0.1

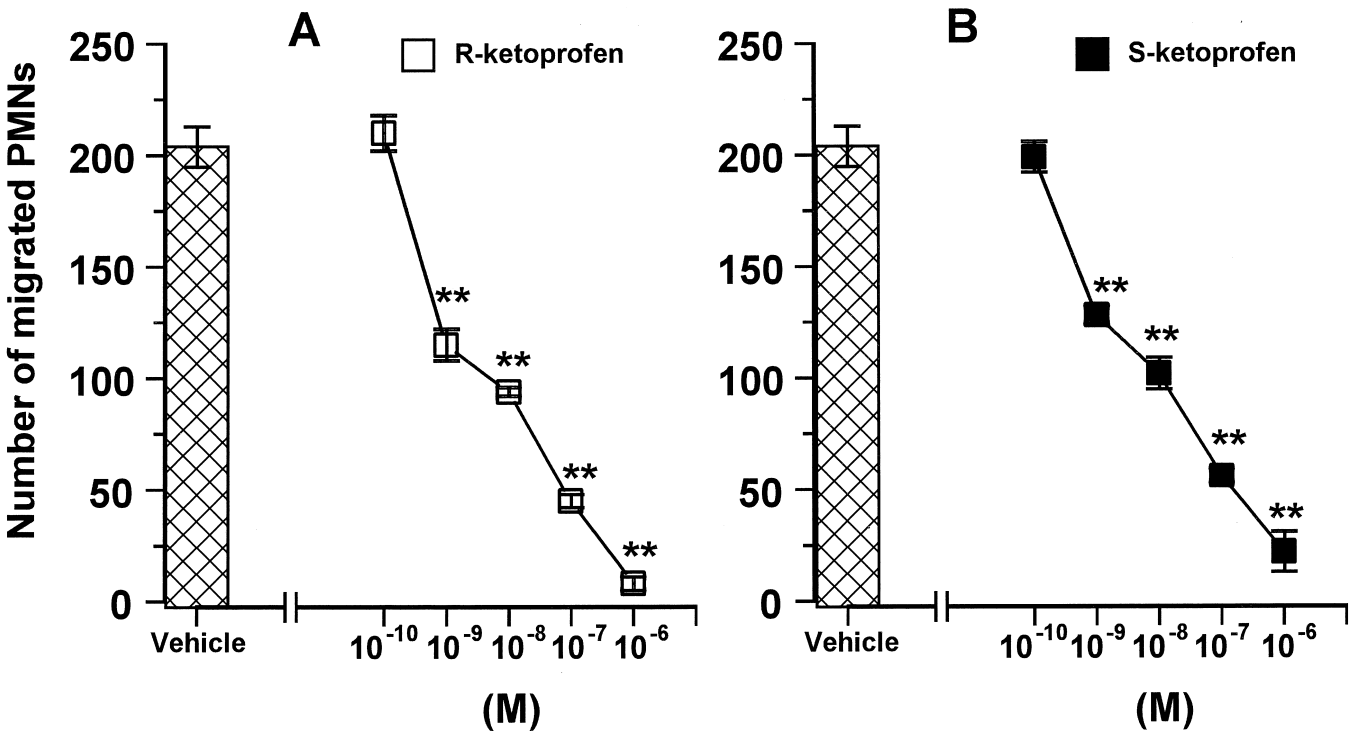

Fig. 1. Effect of R- and S-ketoprofen on IL-8-induced PMN migration. PMNs were preincubated at 37° for 15 min with vehicle (☒) or increasing concentrations of R-ketoprofen (□; A) or S-ketoprofen (■; B). PMNs were then tested for their ability to migrate in response to 10 ng/mL IL-8. PMN migration was determined as described in section 2. Data are expressed as mean values ± SD of three independent experiments. Spontaneous PMN migration was 16 ± 3. Statistical analysis was performed by Student's *t* test and Mann–Whitney *U* test. Statistical threshold was set at $P < 0.05$. $^{**}P < 0.01$ vs IL-8 alone (vehicle pretreated) group by Student's *t* test and Mann–Whitney *U* test.

and 1 μM) reduced MGSA/GROα chemotaxis (64% and 73% inhibition of PMN migration by R- and S-isomer at 1 μM, respectively). In the absence of chemokine stimulation, ketoprofen isomers alone were unable to modify PMN spontaneous migration over a wide range of concentrations (data not shown).

Next, we have evaluated the effect of additional 2-arylpropionic acids as well as other NSAIDs on IL-8-induced PMN chemotaxis. The effect of diclofenac, indomethacin, flurbiprofen and ibuprofen was investigated at 10 nM. Ketoprofen racemate was included in the experiment as reference drug. All drugs strongly reduced PMN migration similarly to ketoprofen (data not shown). Comparable results were also obtained after PMN pretreatment with 10 nM of R- and S-isomers of flurbiprofen and ibuprofen (data not shown).

To evaluate whether ketoprofen enantiomers could exert an inhibitory activity on IL-8-mediated biological activities other than chemotaxis, their effect on IL-8-induced PMN degranulation was investigated. Elastase activity in cell-free supernatants was evaluated as a marker of azurophilic granule release. R- and S-isomers weakly reduced PMN elastase release induced by an optimal concentration of IL-8 (50 ng/ml) [30], being the effect significant only at 1 μM (Δ OD $\times$ 10^3/min: 20.4 ± 1.4, 15.9 ± 0.5*, and 17.2 ± 1* in vehicle, R-ketoprofen-, and S-ketoprofen-pretreated PMNs, respectively; mean ± SD of three independent experiments;

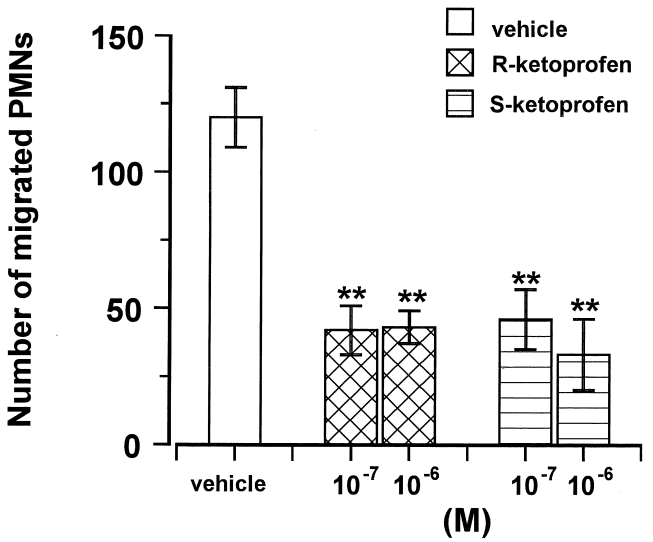

Fig. 2. Inhibition of MGSA/GROα chemotaxis by ketoprofen isomers. PMNs were preincubated at 37° for 15 min with vehicle (□) or two different concentrations (10^{-7} and 10^{-6} M) of R-ketoprofen (☒) or S-ketoprofen (⊟). Then the cells were seeded in the upper compartment of Boyden chamber. MGSA/GROα (100 ng/mL) was added in the lower compartment. PMN migration was determined as described in section 2. Data are expressed as mean values ± SD of three independent experiments. Spontaneous PMN migration was 13 ± 4. $^{**}P < 0.01$ vs IL-8 alone (vehicle pretreated) group by Student's *t* test and Mann–Whitney *U* test.

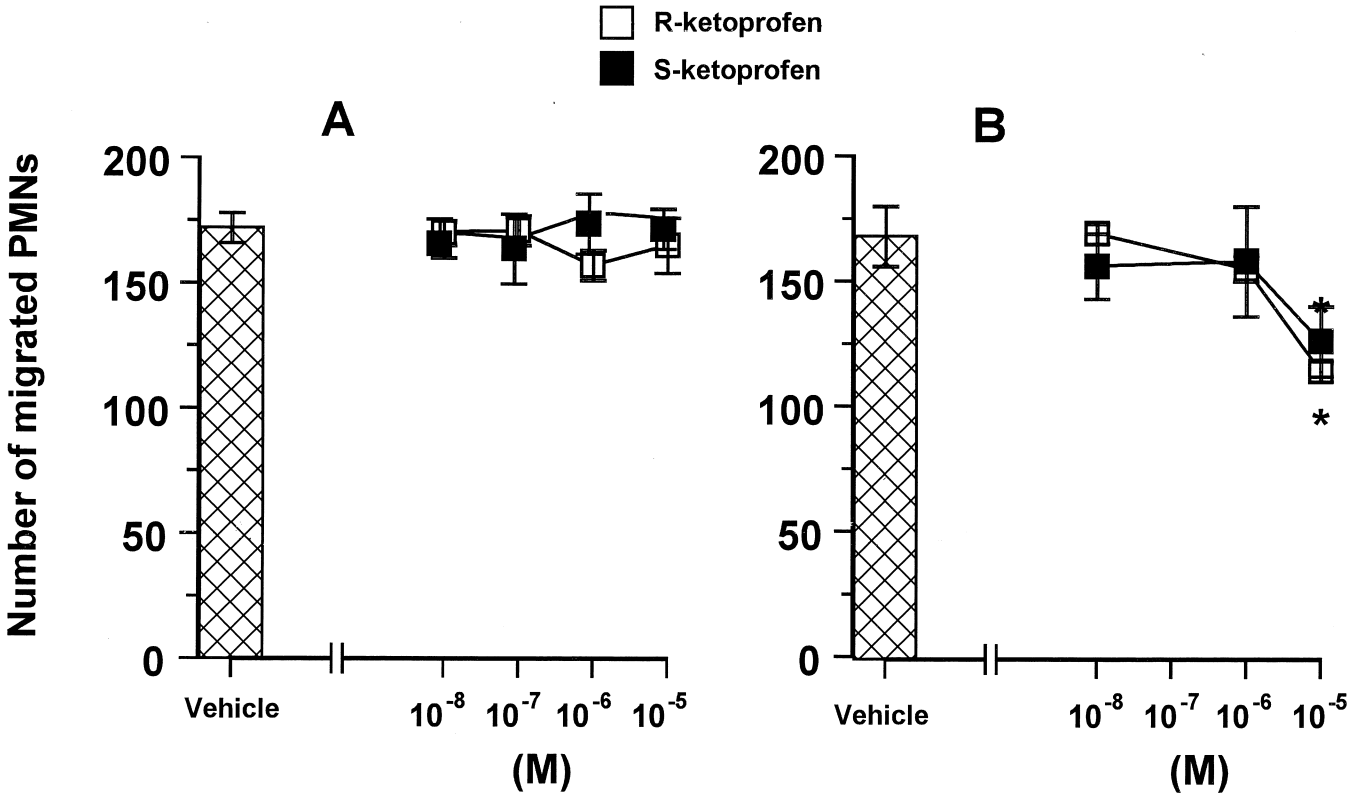

Fig. 3. Effect of R- and S-ketoprofen on C5a and fMLP-induced PMN migration. PMNs were preincubated at 37° for 15 min with vehicle (☒) or with increasing concentrations of R-ketoprofen (□) or S-ketoprofen (■). PMNs were then tested for their ability to migrate in response to fMLP (10^{-7} M, panel A) or C5a (10^{-9} M, panel B). Data are expressed as mean values ± SD of three independent experiments. Spontaneous PMN migration was 20 ± 5. *$P < 0.05$ versus stimulus alone (vehicle pretreated) groups by Student's *t* test and Mann-Whitney *U* test.

*$P < 0.05$ vs vehicle group by Student's *t* test). Comparable reduction of elastase release by R and S-ketoprofen was also observed after PMN stimulation with a suboptimal concentration of IL-8 (10 ng/mL; Δ OD × 10^3/min: 14.7 ± 0.6, 12.2 ± 0.7, and 11 ± 0.3 in vehicle, 1 μM R-ketoprofen, and 1 μM S-ketoprofen pretreated PMNs, respectively).

3.2. Effect on leukocyte activities induced by fMLP, C5a, and MCP-1

To investigate whether inhibition of PMN migration could be restricted to IL-8, we examined the effect of R and S-ketoprofen on fMLP and C5a-induced chemotaxis. The effect of ketoprofen isomers was evaluated in the same range of concentrations affecting IL-8 chemotaxis. Results in Fig. 3A show that ketoprofen isomers did not affect PMN migration induce by fMLP over a wide range of concentrations. Similarly, R- and S-enantiomers significantly reduced C5a chemotaxis only at the highest concentration tested (10 μM; Fig. 3B; 32% and 25% of inhibition by R and S-ketoprofen, respectively). In addition, ketoprofen isomers did not reduce fMLP and C5a-induced elastase release over the whole range of concentrations tested (data not shown).

Finally, the effect on monocyte migration was examined. R- and S-isomers (0.1 or 1 μM) did not affect monocyte migration induced by MCP-1 (25 ng/ml), a monocyte chemokine belonging to the C-C chemokine family [9; data not shown].

3.3. Inhibition of IL-8-induced intracellular mediators

$[Ca^{2+}]_i$ increase represents a key event in IL-8-induced PMN activation. Table 1 shows that a 15 min preincubation with ketoprofen isomers reduced the peak of $[Ca^{2+}]_i$ induced by IL-8 (50 ng/ml) in PMNs. R- and S-ketoprofen reduced elevation of $[Ca^{2+}]_i$ induced by IL-8 starting at 10 nM concentration. The number of PMNs with increased $[Ca^{2+}]_i$ after IL-8 treatment was significantly inhibited by ketoprofen enantiomers at 1 μM. On the contrary, R and S-isomers did not reduce increase of $[Ca^{2+}]_i$ in PMNs stimulated by fMLP over the concentration range tested (1 nM-1 μM; Table 1). Fig. 4 shows traces of $[Ca^{2+}]_i$ in single PMNs stimulated with IL-8 or fMLP in the presence or absence of ketoprofen isomers. In the absence of agonist stimulation, ketoprofen enantiomers alone were unable to modify PMN basal $[Ca^{2+}]_i$ (data not shown).

ERKs are activated by phosphorylation of tyrosine/threonine residues [31,32]. Since it has been reported that activation of ERKs by chemotactic agents might be involved

Table 1
Effect of ketoprofen isomers on IL-8 or fMLP-induced increase of $[Ca^{2+}]_i$ in single adherent PMNs

[M]	IL-8						fMLP					
	Treatment						Treatment					
	R-ketoprofen			S-ketoprofen			R-ketoprofen			S-ketoprofen		
	$[Ca^{2+}]_i$	N	RF	$[Ca^{2+}]_i$	N	RF	$[Ca^{2+}]_i$	N	RF	$[Ca^{2+}]_i$	N	RF
vehicle	302 ± 11	70	89	302 ± 11	70	89	204 ± 7	32	100	204 ± 7	32	100
10^{-9}	335 ± 29	8	100	289 ± 19	11	82	ND			ND		
10^{-8}	188 ± 8*	36	86	183 ± 5*	29	76	204 ± 21	7	100	ND		
10^{-7}	193 ± 10*	19	89	199 ± 12*	7	73	199 ± 11	15	100	201 ± 9	28	96
10^{-6}	182 ± 15*	22	50*	164 ± 18*	7	43*	193 ± 6	19	100	214 ± 13	8	100

PMNs were preincubated at 37° for 15 min with vehicle or increasing concentrations of ketoprofen isomers. PMNs were tested for their ability to increase $[Ca^{2+}]_i$ in response to IL-8 (50 ng/mL) or fMLP (10^{-7} M). Cells were considered responsive when the stimulus-induced increase of $[Ca^{2+}]_i$ was more than 30% over baseline (normalized to 100%). Mean $[Ca^{2+}]_i$ increase is the mean ± SEM of values of responsive cells. Data are expressed both as % above basal $[Ca^{2+}]_i$ and as % of responsive cells (RF, response frequency). The table reports comulative data of 7–70 cells (N, total number of cells) analyzed. Basal $[Ca^{2+}]_i$ in resting adherent PMNs was 125 ± 5 nM.

* $P < 0.05$ vs IL-8 alone (vehicle pretreated) group by analysis of variance (ANOVA), Student's multiple comparison *t* test and Dunnett's multiple range test. ND, not determined.

in IL-8-induced activation of PMNs [33], we investigated the effect of R- and S-isomers on IL-8-mediated ERK-1/2 activation by immunoblotting with antiserum specific for phosphorylated ERK-1/2. As shown in Fig. 5 (upper panel), PMN stimulation with 100 nM fMLP or 100 ng/mL of IL-8 (at 37° for 3 min) caused a significant increase in phosphorylation of ERK-2. Preincubation of PMNs with R- or S-ketoprofen (0.1 μM) inhibited ERK-2 phosphorylation in

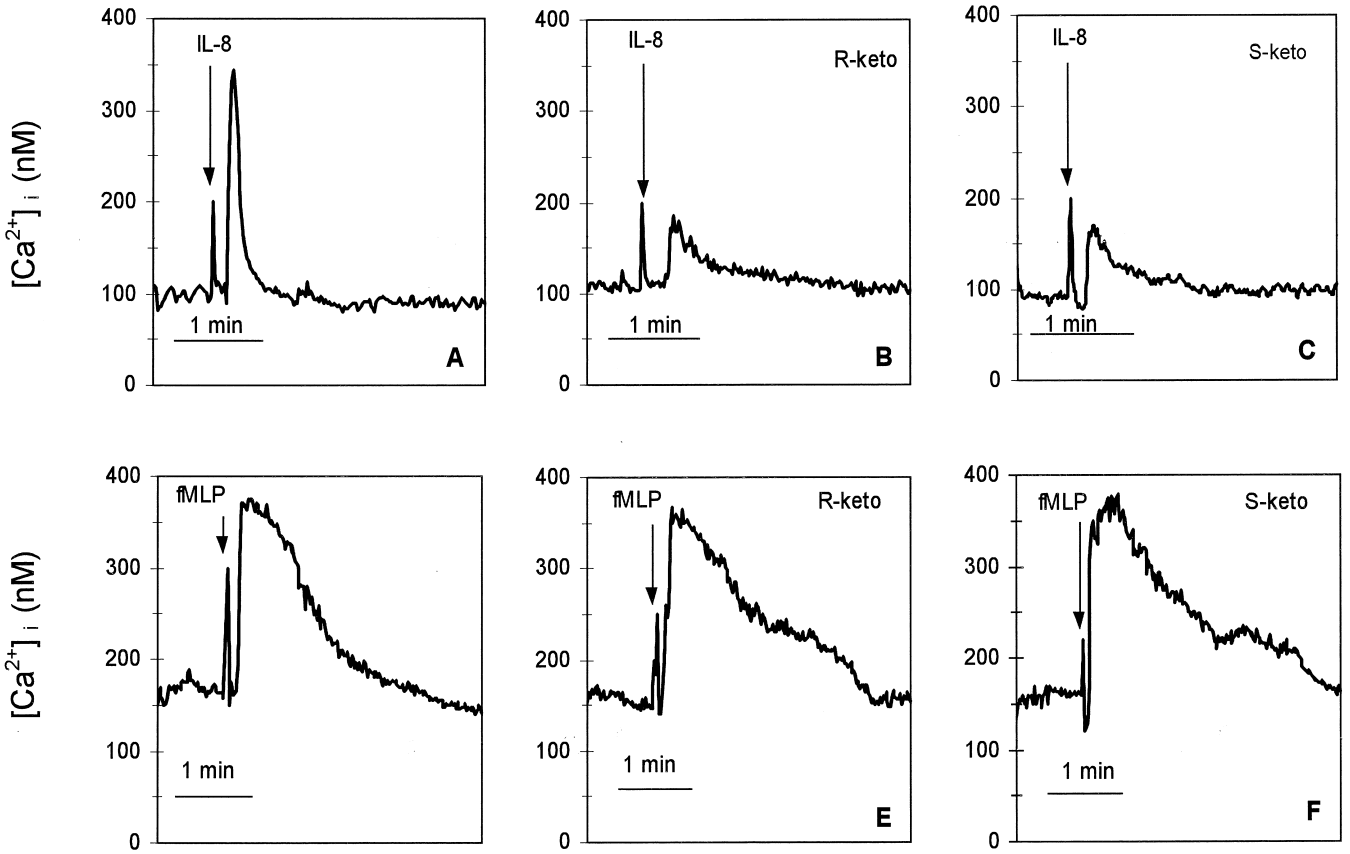

Fig. 4. Inhibition of R- and S-ketoprofen on IL-8-induced changes of $[Ca^{2+}]_i$ in adherent human PMNs. Traces represent levels of $[Ca^{2+}]_i$ in single adherent PMNs in response to IL-8 (50 ng/ml) after a 15 min preincubation with vehicle (panel A), R-ketoprofen (10^{-8} M, panel B) and S- ketoprofen (10^{-8} M, panel C). Panels D, E, and F show the $[Ca^{2+}]_i$ response to fMLP (10^{-7} M) in PMN pretreated with vehicle, R-ketoprofen and S-ketoprofen, respectively. Traces are representative of 7 to 70 cells. Arrows indicate the addition of agents to the bathing medium, which causes a spike in the trace due to exposure to light.

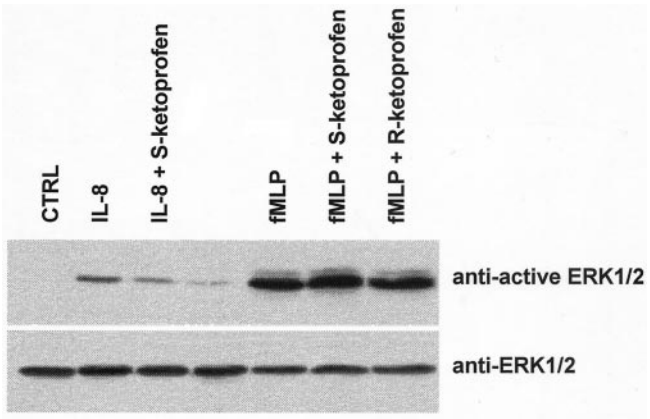

Fig. 5. Inhibition of R- and S-ketoprofen on IL-8-induced ERK1/2 phosphorylation. PMNs were preincubated at 37° for 30 min with the vehicle or 10^{-7} M R-ketoprofen or S-ketoprofen, and stimulated with 100 ng/ml IL-8 for 3 min. Upper panel. Results obtained with the anti-active ERK1/2. Lower panel. The same filter was probed with an anti-total ERK1/2. Results of one of two similar experiments are shown.

response to IL-8, being R-ketoprofen even more potent than S-isomer. Conversely, ketoprofen isomers were unable to reduce ERK-2 phosphorylation in fMLP-stimulated PMN. None of the agents tested affected the amount of ERK-2 present in the lysates (Fig. 5, lower panel) as assessed by an anti-ERK-1/2 antibody recognizing ERK 1/2 irrespective of the phosphorylation state.

Finally, the effect of ketoprofen isomers on IL-8 receptor binding was explored. PMN preincubation with R- or S-ketoprofen (1 μM) at 37° for 15 min did not show a significant change in IL-8 receptor number (12207 $\pm$ 2330, 14609 $\pm$ 2828, and 13287 $\pm$ 1657 in vehicle, R-ketoprofen and S-ketoprofen-pretreated PMN, respectively; mean $\pm$ SD of six different donors) or affinity (Kd 2.4 $\pm$ 0.6 $\times$ 10^{-10} M, 3.4 $\pm$ 1.6 $\times$ 10^{-10} M and 2.6 $\pm$ 0.5 $\times$ 10^{-10} M, respectively).

4. Discussion

This article shows that R- and S-isomers of ketoprofen are potent and specific inhibitors of IL-8-induced chemotaxis of human PMNs. Concentrations of R- and S-ketoprofen isomers found efficacious in inhibiting IL-8-induced chemotaxis, are within the range of plasma levels found in patients given ketoprofen racemate. Inhibition of PMN chemotaxis is restricted to IL-8, as C5a- and fMLP-induced chemotaxis of PMNs is unaffected by R- and S-ketoprofen isomers. Similarly, monocyte chemotaxis induced by MCP-1, a C-C chemokine, is not inhibited by ketoprofen isomers. Ketoprofen isomers-induced inhibition of PMN activation by IL-8 appears to be restricted to chemotaxis, as IL-8-induced degranulation was not affected. In addition to ketoprofen isomers, other NSAIDs, were also found able to inhibit IL-8-induced chemotaxis of human PMNs.

Previous data supported that inhibition of PMN activation by different NSAIDs may contribute to the anti-inflammatory properties of these drugs [2–7,34]. However, only fMLP and phorbol myristate acetate-induced activation were investigated and generally drug concentrations were significantly higher than those found in patients in a therapeutic setting [3,4,6]. Our data are especially relevant in view of the physiopathological role of IL-8. Indeed, it was suggested that chemotactic recruitment of PMNs into synovial fluid by IL-8 could play a crucial role in the development and maintenance of arthritic diseases [11,35] and our data were obtained in a range of drug concentrations comparable to those detected in synovial fluid of arthritic patients treated with therapeutic doses of NSAIDs [36–37].

Inhibition of IL-8 chemotaxis by R- and S-ketoprofen is not attributable to PG synthesis inhibition, as suggested by the finding that R- and S-ketoprofen equally inhibited IL-8 chemotaxis whereas R-ketoprofen was at least 100-fold less effective than the S-isomer on COX inhibition [17–19]. Similarly, R- and S-isomers of flurbiprofen and ibuprofen equally reduced PMN migration, further stressing the involvement of a COX-independent mechanism in IL-8 chemotaxis inhibition.

Several lines of evidence suggest that COX blockade could only partially contribute to the therapeutic effects NSAIDs [1]. For instance, the analgesic and anti-inflammatory effectiveness of many NSAIDs in current use does not correlate very well with their potency as PG synthesis inhibitors. Accordingly, we have recently reported that R-ketoprofen accounts for most of the analgesic activity of ketoprofen [17] and the analgesic efficacy of R-flurbiprofen was shown to be comparable to that of the S-isomer [18]. Furthermore, there is no correlation between the low doses of aspirin that inhibit PG synthesis in vitro and the higher doses required to exert the anti-inflammatory effect in vivo [38]. Finally, it was reported that stable PGs inhibit PMN activities in vitro [39–41] and have anti-inflammatory activity in animal models of arthritis [42]. Our data suggest that in addition to PG synthesis inhibition, NSAIDs may exert their anti-inflammatory activity also by means of IL-8-induced PMN recruitment inhibition.

Reduction of IL-8 migration by ketoprofen isomers was paralleled by inhibition of $[Ca^{2+}]_i$ increase and ERK-2 activation induced by IL-8, two early signaling events that are strictly associated with PMN functions. Accordingly with the lack of inhibition of fMLP-induced chemotaxis, ketoprofen isomers did not affect $[Ca^{2+}]_i$ increase and ERK-2 activation in fMLP-stimulated PMNs. PMN activation is at least in part dependent on the rise in $[Ca^{2+}]_i$. Inhibition of $[Ca^{2+}]_i$ influx by inorganic blockers of non-selective cation channel suppressed PMN functions [43], although recent experiments with phospholipase C-beta2-null mice suggest that $[Ca^{2+}]_i$ increase could not be indispensable for PMN migration [44]. ERK is considered a key mediator of PMN adhesion and chemotaxis [7,33]. The finding that a marked reduction of adhesion was observed in

PMNs from normal volunteers after NSAID medication is in keeping with the concept that inhibition of IL-8 migration by ketoprofen isomers could correlate with reduction of ERK activation [45]. We found that R-ketoprofen is more potent than S-ketoprofen in inhibiting ERK activation. Since ERK-kinases activate intracellular transcription factors including c-Fos and transcription nuclear factor-κB [46], the differential inhibitory effect of R-ketoprofen on ERK-2 activation could contribute to the anti-inflammatory activity of this isomer. In agreement with this hypothesis, it was reported that the analgesic effect of ketoprofen could be related with the inhibition of spinal c-*Fos* protein [47] and that, as previously discussed, R-ketoprofen accounts for most of the analgesic activity of ketoprofen [17]. In addition, it is known that R-ibuprofen reduces the activation of transcription nuclear factor-κB, a crucial mediator of inflammatory cytokines gene expression [48].

Although ketoprofen isomers reduced PMN intracellular mediators reported to be crucial for IL-8 activities, we have not defined the molecular mechanism of the selective inhibitory effect of ketoprofen isomers on IL-8 chemotaxis. Since ketoprofen isomers did not change neither the number nor the affinity of IL-8 receptors, it is conceivable that ketoprofen isomers may act at a step downstream receptor binding but upstream $[Ca^{2+}]_i$ increase and ERK activation. In agreement with this hypothesis, it was reported that NSAIDs could inhibit activation of G-proteins associated with seven-transmembrane receptors and thereby uncouple post-receptor signaling events [49]. In addition, differences in IL-8 intracellular receptor sequence involved in G-protein interaction [50] with the corresponding amino acids of fMLP receptor [51] may justify the selective inhibition of ketoprofen isomers on IL-8 chemotaxis. On the other hand, since it was reported that IL-8 and fMLP regulate ERK activation by different pathways [52,53] we can not exclude the hypothesis of a site of action of ketoprofen isomers downstream G-proteins activation.

In summary, we found that R and S ketoprofen isomers inhibit IL-8-induced chemotaxis. Inhibition of IL-8 chemotaxis was independent from PG synthesis inhibition and was paralleled by selective inhibition of PMN intracellular mediators reported to be crucial for IL-8 activities. The fact that ketoprofen isomers, as well as drugs belonging to several classes of NSAIDs, blocked IL-8 chemotaxis in a therapeutical range of concentrations could represent a new clinical target for ketoprofen isomers and, in fact, contribute to the anti-inflammatory activity of NSAIDs.

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