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RESEARCH PAPER

Neutrophil migration towards C5a and CXCL8 is prevented by non-steroidal anti-inflammatory drugs via inhibition of different pathways

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BACKGROUND AND PURPOSE

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to induce PG-independent anti-inflammatory actions. Here, we investigated the role of three different NSAIDs (naproxen, ibuprofen and oxaprozin) on neutrophil responses to CXCL8 and C5a.

EXPERIMENTAL APPROACH

Human neutrophils were isolated from healthy volunteers by dextran and Ficoll-Hypaque density gradients. Neutrophils were pre-incubated with different concentrations (1–100 μM) of NSAIDs or kinase inhibitors. Neutrophil degranulation into supernatants was tested by ELISA and zymography. Neutrophil chemotaxis was determined using Boyden chambers. F-actin polymerization was determined by Alexa-Fluor 488-conjugated phalloidin fluorescent assay. Integrin expression was assessed by flow cytometry. The phosphorylation of intracellular kinases was studied by Western blot.

KEY RESULTS

Pretreatment with NSAIDs did not affect neutrophil degranulation, but inhibited neutrophil migration and polymerization of F-actin, in response to CXCL8 and C5a. Pretreatment with different NSAIDs prevented C5a-induced integrin (CD11b) up-regulation, while only ibuprofen reduced CXCL8-induced CD11b up-regulation. Pre-incubation with naproxen or oxaprozin, but not ibuprofen, inhibited the PI3K/Akt-dependent chemotactic pathways. Both endogenous (released in cell supernatants) or exogenous (added to cell cultures) PGE_2 did not affect C5a- or CXCL8-induced activities. Short-term incubation with NSAIDs did not affect neutrophil PGE_2 release.

CONCLUSION AND IMPLICATIONS

Treatment with NSAIDs reduced C5a- and CXCL8-induced neutrophil migration and F-actin polymerization via different mechanisms. Inhibition by ibuprofen was associated with integrin down-regulation, while naproxen and oxaprozin blocked the PI3K/Akt pathway. Both NSAID actions were independent of COX inhibition and PGE_2 release.

Abbreviations

CV, coefficient of variation; NSAIDs, non-steroidal anti-inflammatory drugs; MFI, mean fluorescence intensity; MPO, myeloperoxidase; PE, phycoerythrine

Introduction

Neutrophil recruitment and degranulation within inflammatory tissues is an essential component of host defence against microbial infections (Henson and Johnston, 1987). However, such responses could also cause tissue injury (Ottonello *et al.*, 1998), as activated neutrophils release large amounts of cytotoxic enzymes, such as MMP-8, MMP-9 neutrophil elastase and myeloperoxidase (MPO) that digest collagen and alter tissue structure (Dallegri and Ottonello, 1997; Borregaard *et al.*, 2007; enzyme nomenclature follows Alexander *et al.*, 2013). Neutrophil infiltration has been described as a key pathophysiological event in several inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease and, more recently, also atherosclerosis (Montecucco *et al.*, 2010; Khandpur *et al.*, 2013; Koelink *et al.*, 2014). Although the properties of the infiltrating neutrophils are still a matter for debate (Ryan *et al.*, 2013), the selective pharmacological control of neutrophil-mediated inflammation has been proposed as a promising strategy to reduce tissue injury (Copin *et al.*, 2013). Non-steroidal anti-inflammatory drugs (NSAIDs) have been recently shown to directly target leukocyte-mediated inflammation, independently of PG production (Angelis-Stoforidis *et al.*, 1998; Ottonello *et al.*, 2009). Among different NSAIDs, we recently focused on oxaprozin (4,5-diphenyl-2-oxazolepropionic acid), showing that human monocyte survival was reduced *in vitro* by this drug (Ottonello *et al.*, 2009). Interestingly, oxaprozin was previously shown to scavenge reactive oxygen species and reactive nitrogen species in cultured neutrophils, compared with other more potent NSAIDs (Fernandes *et al.*, 2004). In the present study, we aimed at investigating the potentially selective role of different NSAIDs (ibuprofen, naproxen and oxaprozin) on neutrophil degranulation, migration and actin polymerization in response to C5a and CXCL8, two known mediators of inflammation (Jose *et al.*, 1990; Kraan *et al.*, 2001; Montecucco *et al.*, 2010). The potential NSAID-mediated modulation of intracellular pathways (Perianayagam *et al.*, 2002; Riedemann *et al.*, 2004) and integrin CD11b expression, two mechanisms previously shown to be involved in neutrophil migration (Montecucco *et al.*, 2008; 2009b), was also assessed.

Methods

Isolation of human primary neutrophils

This protocol was approved by the local Ethics Committee of San Martino Hospital in Genoa (Italy) and conformed to the principles outlined in the Declaration of Helsinki. Neutrophils were isolated from human heparinized (heparin 10 U mL^{-1}; Vister, Pfizer Italia, Borgo San Michele, Latina, Italy) venous blood from healthy volunteers (age 24–48 years old) after written informed consent. Neutrophils were prepared by Dextran 70.000 (Plander, Fresenius Kabi Italia, Verona, Italy) sedimentation, followed by centrifugation (400× *g*, 30 min) on a Ficoll-Hypaque density gradient. Plasma and the mononuclear cell ring were discarded and contaminant erythrocytes were removed by hypotonic lysis. Neutrophils were then resuspended in the migration assay medium [HBSS; EuroClone, Wetherby West, UK, mixed with Dulbecco's PBS (HBSS-PBS 3:1; EuroClone)] containing 1 mg mL^{-1} BSA (Sigma Chemical, St. Louis, MO, USA). Neutrophils were on average 97% pure, as determined by morphological analysis of May-Grünwald Giemsa (Merck, Darmstadt, Germany) stained cytopreparations.

Detection of neutrophil granule products in cell supernatants

After isolation, 5×10^5 neutrophils were pre-incubated 1 h with control medium alone or in the presence of naproxen, ibuprofen (Sigma) or oxaprozin (Helsinn SA, Pambio-Noranco, Switzerland). All NSAIDs were used at 50 μM. Then cells were stimulated in the presence or absence of 1 nM CXCL8, 1 nM C5a or 10 ng mL^{-1} phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich, Buchs, Switzerland) as positive control, for 30 min. The cell supernatants were collected and assessed for the levels of MMP-9, MMP-8 and MPO by colorimetric ELISA (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions. The lower limits of detection for ELISAs were 0.156 ng mL^{-1} for MMP-8, 0.312 ng mL^{-1} for MMP-9 and 1.56 ng mL^{-1} for MPO. Mean intra- and inter-assay coefficients of variation (CV) were below 8% for all markers (Montecucco *et al.*, 2008).

Pro-MMP-9 zymography

The same neutrophil supernatants collected for the ELISA determination of degranulation product were also tested for pro-MMP-9 zymographic activity. Equal amounts of cell supernatants (30 μL) and 1 ng of recombinant pro-MMP-9 standard (Calbiochem, Lucern, Switzerland) were loaded on gels 9% SDS-polyacrylamide copolymerized with gelatine (Sigma) and in the absence of reducing agents. Then gels were rinsed and stained with Coomassie Blue R-250. Zymographic results were expressed as pro-MMP-9 proteolytic activity and calculated on the basis of the following formula: supernatant pro-MMP-9 = $(I_{obs} / I_{std}) \times W_{std}$, where I_{obs} and I_{std} are intensities of gelatinolytic areas produced in gels by samples and by standard pro-MMP-9, and W_{std} is the weight (1 ng) of standard pro-MMP-9 loaded onto the gel. Pro-MMP-9 activity was expressed as ng·mL^{-1}. Gelatinolytic bands were measured with a gel analysis system (GeneGenius, Syngene, Cambridge, UK).

Modified Boyden chamber migration assay

Neutrophils were pre-incubated for 1 h with or without different concentrations (1–100 μM) of ibuprofen, naproxen or oxaprozin. In parallel experiments, neutrophils were pretreated for 1 h in the presence or absence of intracellular kinase inhibitors [10 μM LY294002 (PI3K inhibitor; Sigma), 40 μM 1L-6-hydroxymethyl-*chiro*-inositol 2[(R)-2-*O*-methyl-3*O*-octadecylcaromate (Akt inhibitor; Calbiochem, San Diego, CA, USA), 25 μM PD98059 (MEK inhibitor; Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA), 1 μM SB203580 (p38 MAPK inhibitor; Biomol Research Laboratories, Inc.)], as previously reported (Montecucco *et al.*, 2008). In addition, some experiments pretreating cells for 1 h with 50 pg mL^{-1} PGE_2 (Sigma-Aldrich) with or without 50 μM of ibuprofen, naproxen or oxaprozin were also performed. Then cells were washed with the migration medium and tested for migration in a 48-well microchemotaxis chamber (Neuro Probe Inc., Gaithersburg, MD, USA) using a 3 μm pore size, 150-μm-thick cellulose ester filter (Neuro Probe, Inc.). The lower wells of the chemotaxis chambers were filled with control medium alone, 1 nM human recombinant CXCL8 (BioSource International, Camarillo, CA, USA) or 1 nM human recombinant complement C5a. The upper wells were filled with 1×10^5 neutrophils suspended in 50 μL. After incubation (45 min, 37°C, 5% CO_2), the filters were removed, fixed and stained with Harris haematoxylin. After dehydration, the filters were cleared with xylene and mounted in Eukitt (Kindler, Freiburg, Germany). Each condition was performed in duplicate. The migration, as the distance (in μm) travelled by the leading front of cells was measured, without

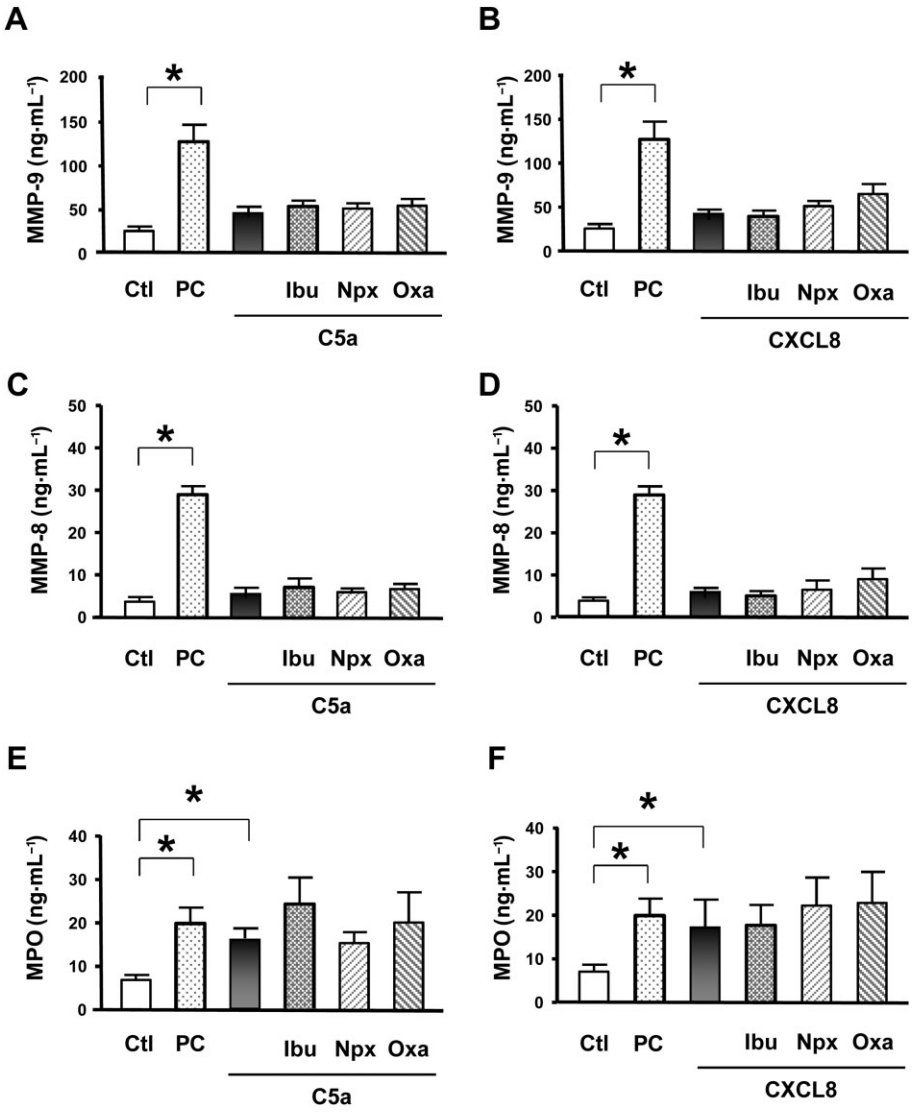

Figure 1

Pretreatment with NSAIDs does not affect C5a- or CXCL8-induced degranulation of human neutrophils. Cells were pretreated for 1 h with control medium (Ctl), 50 μM ibuprofen (Ibu), 50 μM naproxen (Npx) or 50 μM oxaprozin (Oxa). Then the cells were stimulated by 1 nM C5a, 1 nM CXCL8 or 10 ng mL^{-1} PMA [as positive control (PC)] for 30 min. The release of MMP-9 (A and B), MMP-8 (C and D) and MPO (E and F) in cell supernatants was assayed. Data are expressed as mean ± SEM ($n = 4$). *$P < 0.05$.

knowledge of the treatment groups, at 1000× magnification. Data were expressed as migration towards control medium, C5a or CXCL8.

Determination of the total cellular F-actin

Freshly isolated neutrophils were pretreated for 1 h with or without 50 μM ibuprofen, 50 μM naproxen or 50 μM oxaprozin. After this incubation, cells were stimulated with medium alone, 1 nM CXCL8 or 1 nM C5a for additional 30 min. Then neutrophils were prepared by cytospin centrifuge on slides. After fixing and treatment with Triton X 100, cells were stained with Alexa-Fluor 488-conjugated phalloidin. The content of F-actin was determined by fluorescence microscopy (Nikon Optiphot-2; Nikon, Melville, NY, USA), as previously described (Quercioli *et al.*, 2012). Image capturing was performed with a Hamamatsu colour-chilled 3 CCD camera. All images were captured using identical camera settings (time of exposure, brightness, contrast and sharpness) and an appropriated white balance set according to the fluorescence filter. Pictures were acquired and analysed by Image-Pro Plus 4.0 (Media Cybernetics Inc., Rockville, MD, USA). The mean fluorescence density was determined from a linear measurement of individual cell fluorescence in randomly chosen fields of each slide. Results were shown as the mean ± SEM of the fluorescence densities (expressed as the relative intensity of each field for each individual slide).

Neutrophil membrane integrity assay and morphological analysis

Neutrophils were pretreated for 1 h with or without 50 μM ibuprofen, 50 μM naproxen or 50 μM oxaprozin. After washing, cells were resuspended in migration medium and cell viability was determined at 1 and 2 h of incubation.

Cells (4×10^4 in 0.1 mL) were mixed with 0.05 mL of staining solution (2 μg mL^{-1} fluorescein diacetate, 4 μg mL^{-1} ethidium bromide in HBSS buffer) and incubated for 10 min at room temperature. Thereafter, a drop of cell suspension was analysed by fluorescence microscopy. Neutrophils with intact membrane (viable cells) appeared as green fluorescent cells, whereas neutrophils with damaged membrane (necrotic cells) became permeable to ethidium bromide, displaying a fluorescent red nucleus. Cell morphology was also analysed on cytopreps by staining with May-Grünwald Giemsa (Merck) (Ottonello *et al.*, 2002).

Flow cytometry

After isolation, neutrophils were pre-incubated for 1 h with or without different concentrations (up to 100 μM) of ibuprofen, naproxen or oxaprozin. Then cells were stimulated with medium alone, 1 nM CXCL8 or 1 nM C5a for an additional 20 min. After washing in PBS, cells were immediately stained with phycoerythrine (PE)-labelled anti-human CD11b antibody, as well as corresponding PE-conjugated isotype control (R&D Systems Europe Ltd, Abingdon, UK) and analysed on a FC500 (Beckman Coulter, Hialeah, FL, USA). CXP software was used for the acquisition and analysis. Autofluorescence levels of sample were measured and subtracted from each analysis. Data were expressed as mean fluorescence intensity (MFI), compared with the expression on cells stimulated with control medium alone (defined as 100%) (Montecucco *et al.*, 2008).

Quantitative analysis of the levels of antigen expression by flow cytometry

The quantitative analysis of CD11b expression on neutrophils was also tested by an established method (Davis *et al.*,

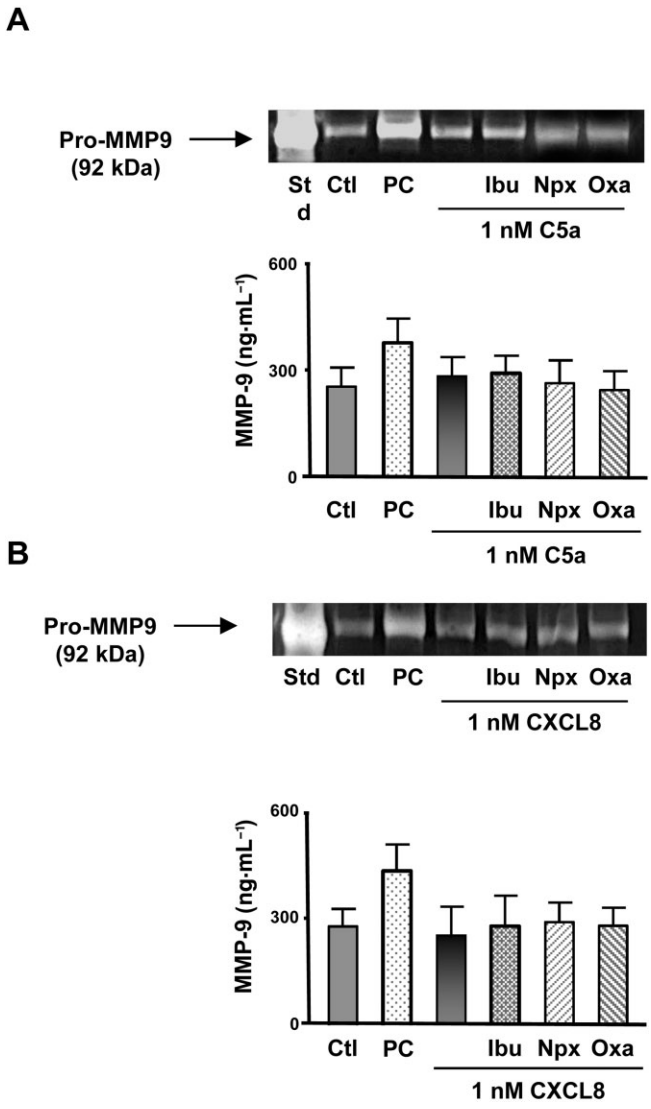

Figure 2

Pretreatment with NSAIDs does not affect MMP-9 activity in neutrophil supernatants. (A) Representative gel (upper part) and quantification (lower part) of pro-MMP-9 zymography of supernatants of cells pretreated with control medium, 50 μM ibuprofen (Ibu), 50 μM naproxen (Npx) or 50 μM oxaprozin (Oxa), and stimulated in the absence (Ctl) or presence of 1 nM C5a. (B) Representative gel (upper part) and quantification (lower part) of pro-MMP-9 zymography of supernatants of cells pretreated with control medium, 50 μM ibuprofen (Ibu), 50 μM naproxen (Npx) or 50 μM oxaprozin (Oxa), and stimulated in the absence (Ctl) or presence of 1 nM CXCL8. Data are expressed as mean ± SEM ($n = 4$). All comparisons between different groups and controls were not statistically significant.

1996; Pannu *et al.*, 2001). QuantiBRITE PE beads (BD Biosciences, Allschwil, Switzerland) were used as calibrators to quantify CD11b fluorescence intensity in units of PE anti-CD11b antibody molecule bound per cell (PE molecules/cell).

Western blot analysis

Freshly isolated human neutrophils (1×10^7 cells mL^{-1}) were pre-incubated for 1 h at 37°C in a humidified atmosphere 5% CO_2 with or without different concentrations (up to 100 μM) of ibuprofen, naproxen or oxaprozin. Then cells were stimulated for an additional 5 min in control medium, 1 nM C5a or 1 nM CXCL8. The incubations were stopped on ice and the cells were centrifuged at 4°C. After removing supernatants, the pellets were lysed in 400 μL of Nonidet P40 buffer (20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 10 mM NaF, 1% Nonidet P40, 10 μg mL^{-1} glycerol, 1 mM PMSF, 10 μg mL^{-1} leupeptin, 10 μg mL^{-1} aprotinin, 0.5 mM Na_3VO_4). Equal amounts of protein (40 μg) for each sample were boiled in loading buffer (62.5 mM Tris-HCl pH 6.8, 0.75% SDS, 3.75% 2-mercaptoethanol, 8.75% glycerol, 0.025% bromophenol

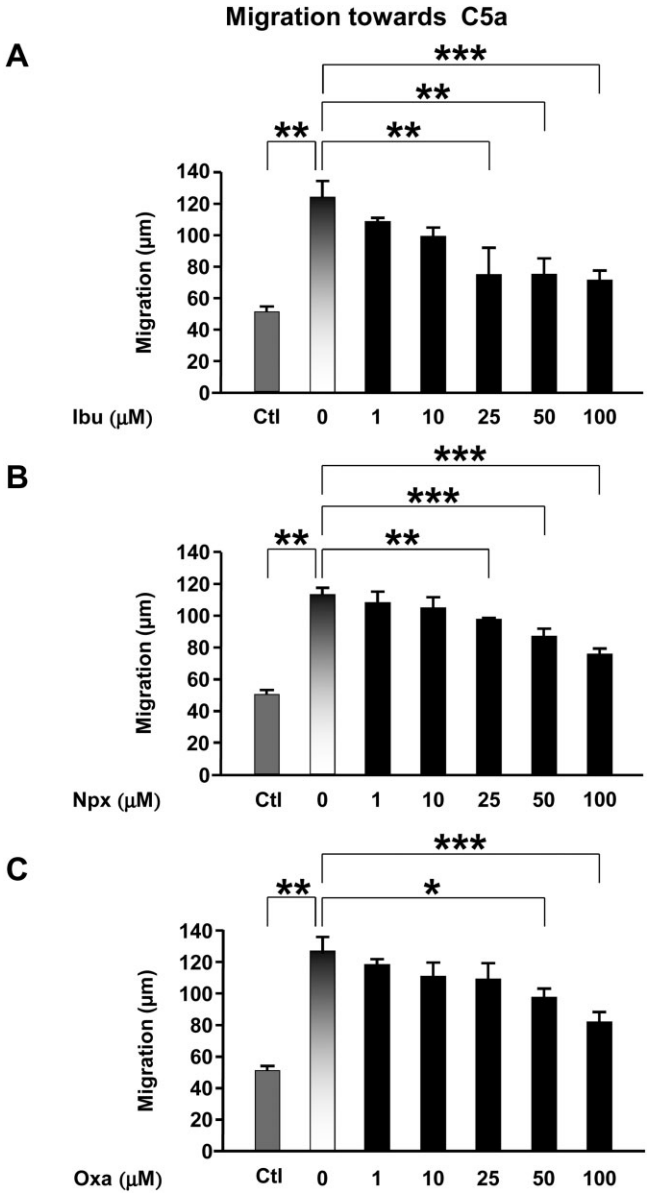

Figure 3

Pre-incubation with NSAIDs blocks neutrophil migration towards 1 nM C5a. (A–C) Migration assay towards 1 nM C5a of neutrophils pretreated for 1 h with medium alone or different concentrations (1–100 μM) of ibuprofen (Ibu) (A), naproxen (Npx) (B) or oxaprozin (Oxa) (C). Results are expressed as migration (μm) and as mean ± SD of three experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

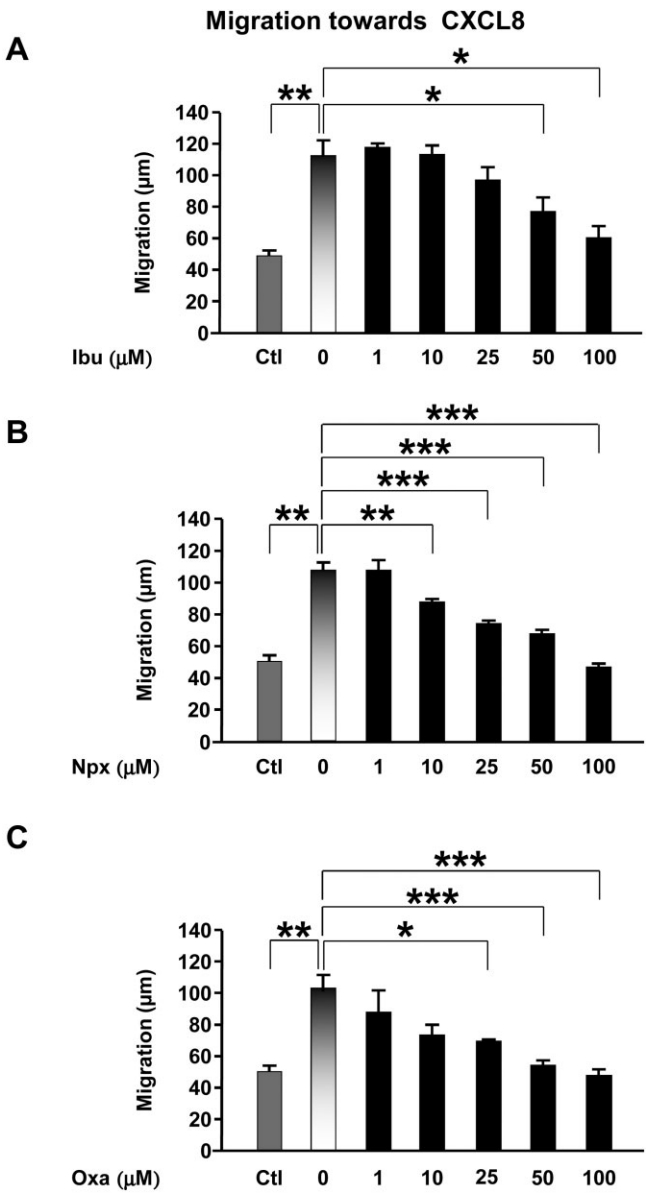

Figure 4

Pre-incubation with NSAIDs blocks neutrophil migration towards 1 nM CXCL8. (A–C) Migration assay towards 1 nM C5a of neutrophils pretreated for 1 h with medium alone or different concentrations (1–100 μM) of ibuprofen (Ibu) (A), naproxen (Npx) (B) or oxaprozin (Oxa) (C). Results are expressed as migration (μm) and as mean ± SD of three experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

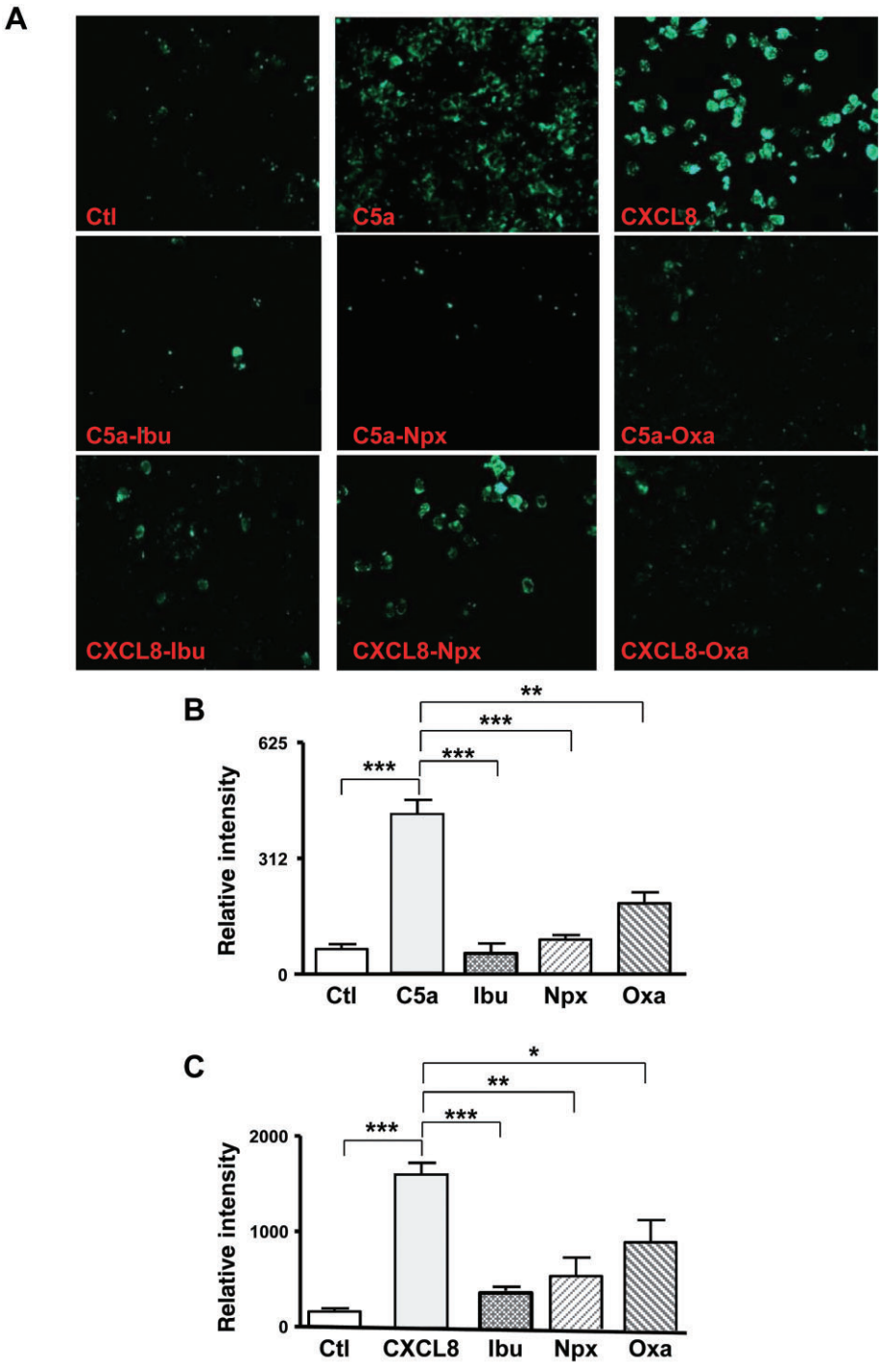

Figure 5

Treatment with NSAIDs blocks F-actin polymerization induced by C5a and CXCL8. (A) Representative pictures of three independent experiments of the determination of F-actin content in neutrophils pretreated with medium alone (Ctl), 50 μM ibuprofen (Ibu), 50 μM naproxen (Npx) or 50 μM oxaprozin (Oxa), and stimulated in the absence (Ctl) or presence of 1 nM C5a or 1 nM CXCL8. (B, C). Quantification of the images. Data are expressed as relative intensities (mean ± SD) of three experiments. *$P < 0.05$; **$P < 0.01$;***$P < 0.001$.

blue) and resolved by 10% SDS-polyacrylamide electrophoresis. Then proteins were transferred on nitrocellulose membrane at 4°C for 45 min. After blocking for 1 h in 5% non-fat dry milk and washing with Tris-buffered saline/Tween 20 (10 mM Tris-base pH 7.4, 154 mM NaCl, 0.05% Tween 20), membranes were incubated with appropriate dilution of anti-phospho-Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-ERK1/2 (R&D Systems), anti-phospho p38 MAPK (R&D Systems) primary antibodies, as well as corresponding secondary antibodies. Blots were developed using the ECL system (Immobilion Western; Millipore, Bedford, MA, USA). Membranes were then stripped, reblocked and reprobed to detect total intracellular kinases, using antibodies against Akt (Santa Cruz Biotechnology), ERK 1/2 (R&D Systems) or p38 MAPK (Santa Cruz Biotechnology). Immunoblots were scanned and quantifications were carried out by Image Quant software version 3.3 (Molecular Dynamics, Sunnyvale, USA). Values of phospho-Akt, phospho-ERK 1/2 and phospho-p38 MAPK (obtained in three different experiments) were normalized to corresponding total amounts of Akt, ERK 1/2 and p38 MAPK, and expressed as percentages of the control medium (defined as 100%). Images of blots were determined by scanning densitometry and quantified with the image analyser system (Syngene, Frederick, MD, USA).

Measurement of PGE_2 levels in neutrophil culture supernatant

Freshly isolated human neutrophils (5×10^6 cells/mL) were pre-incubated for 1 h at 37°C in a humidified atmosphere 5% CO_2 with or without 50 μM ibuprofen, 50 μM naproxen or 50 μM oxaprozin. Then cells were stimulated with medium alone, 1 nM CXCL8, 1 nM C5a or 10 ng mL^{-1} PMA (positive control) (Pouliot *et al.*, 1998) for an additional 30 min. The cell supernatants were collected and assessed for the levels of PGE_2 by colorimetric enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MN, USA), following the manufacturer's instructions. The lower limit of detection was 7.8 pg mL^{-1}. Mean intra- and inter-assay CV were less than 10% for all markers (Hattermann *et al.*, 2007).

Densitometry and statistical analysis

Data were expressed as mean ± SEM or mean ± SD. The Mann–Whitney nonparametric test was used for comparisons of continuous variables between two groups. One-way ANOVA with Bonferroni's post-test was used for multiple group comparison (using GraphPad InStat version 3.05 for Windows XP; GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ (two tailed) were considered statistically significant.

Results

Pretreatment with NSAIDs does not affect C5a- and CXCL8-induced neutrophil degranulation

Human neutrophil degranulation was assessed by measuring the concentrations of different granule products (such as MMP-9, MMP-8 and MPO) in the supernatants (Quercioli *et al.*, 2012). As expected, incubation with PMA (positive control) induced the degranulation of MMP-9 (Figure 1A and B), MMP-8 (Figure 1C and D) and MPO (Figure 1E and F) in cultured neutrophils. However, incubation with C5a or CXCL8 only increased MPO release (Figure 1E and F). Pre-incubation with NSAIDs did not affect either C5a- and CXCL8-induced exocytosis of any granule product measured (Figure 1A–F). As an additional control, pro-MMP-9 gelatinolytic activity in the same neutrophil supernatants was assessed. From the ELISA results, incubation with C5a or CXCL8 did not affect pro-MMP-9 gelatinolytic activity, compared with control medium (Figure 2A and B). Pretreatment with NSAIDs was also confirmed to be ineffective in altering pro-MMP-9 activity (Figure 2A and B).

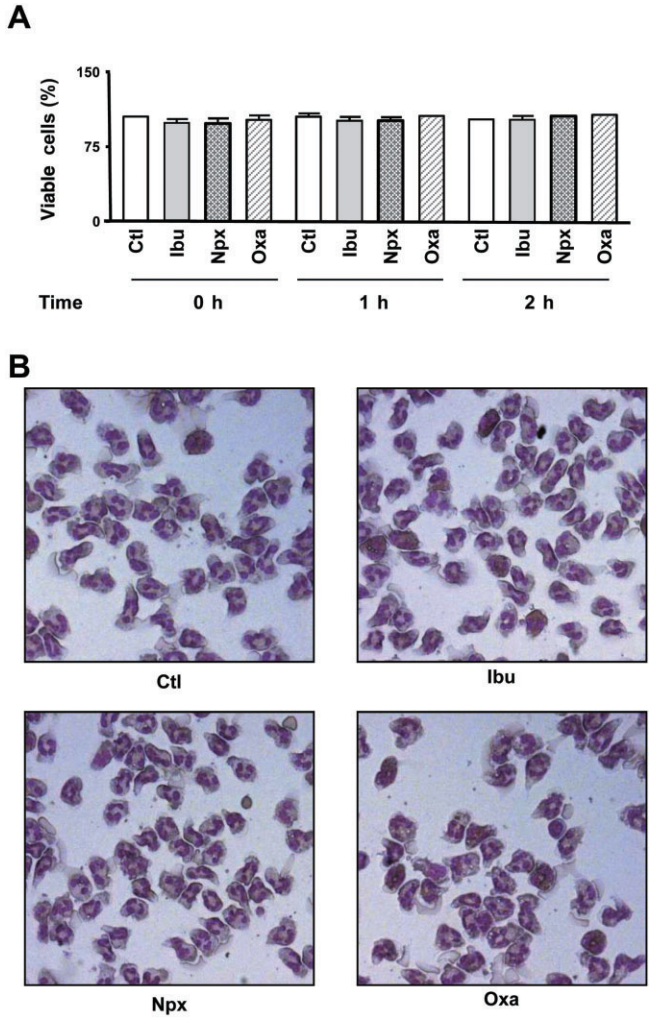

Figure 6

Pretreatment with NSAIDs does not affect cell viability. (A) Determination of cell viability (fluorescein diacetate and ethidium bromide staining) in neutrophils incubated for different periods (up to 2 h) with medium alone (Ctl), 50 μM ibuprofen (Ibu), 50 μM naproxen (Npx) or 50 μM oxaprozin (Oxa). Data are expressed as mean ± SEM ($n = 4$). (B) Representative photograph showing morphological features of neutrophils at 2 h of incubation (May-Grünwald Giemsa staining).

Treatment with NSAIDs prevents neutrophil migration and polymerization of F-actin content in response to C5a and CXCL8

In order to test the potential effects of NSAIDs on neutrophil migration towards the chemoattractants C5a and CXCL8, cells were pretreated with or without different concentrations (1–100 μM) of ibuprofen, naproxen or oxaprozin. All NSAIDs concentration-dependently reduced neutrophil migration towards C5a (Figure 3A–C) and CXCL8 (Figure 4A–C). In addition, we assessed the effects of NSAIDs on polymerization of F-actin, a physiological mechanism known to be associated with neutrophil migration, (Quercioli *et al.*, 2012). As shown in Figure 5A–C, pretreatment with NSAIDs markedly reduced C5a- and CXCL8-mediated polymerization of F-actin, compared with that in cells pre-treated with medium alone.

To control for possible cytotoxicity due to the NSAIDs, cell viability was assessed for all the time points (up to 2 h), covering the incubation periods of different *in vitro* experi-

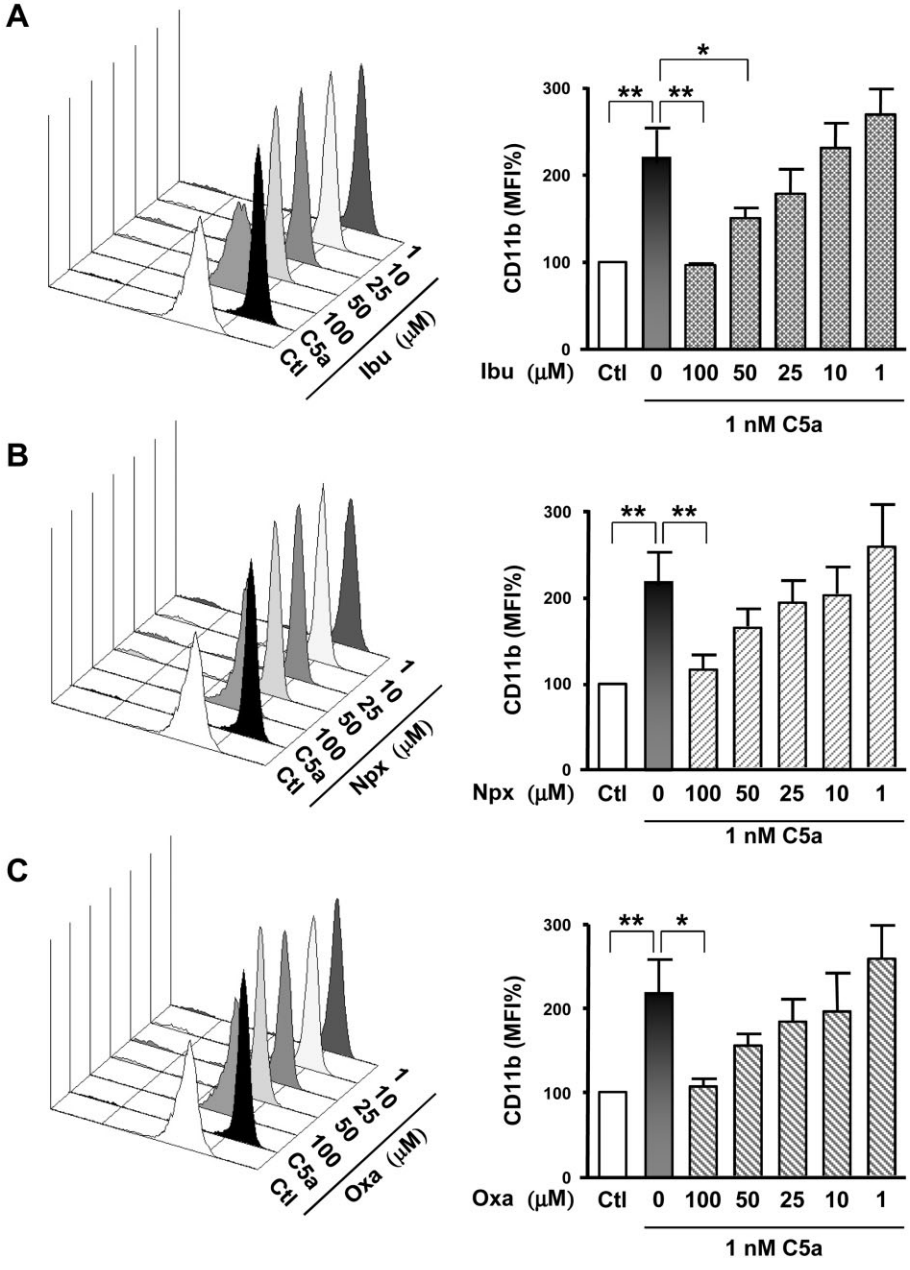

Figure 7

Analysis of CD11b expression (mean fluorescence intensity) on neutrophils pretreated with NSAIDs and stimulated with C5a. (A) Flow cytometry analysis of CD11b expression on human neutrophils cultured for 1 h with or without different concentrations (up to 100 μM) of ibuprofen (Ibu), naproxen (Npx) or oxaprozin (Oxa), and then stimulated with 1 nM C5a for 20 min. (B) Quantification of the flow cytometry diagrams. Data are expressed as mean ± SD of percentages of MFI relative to the non-stimulated control (Ctl) of five experiments: *$P < 0.05$; **$P < 0.01$.

ments in this study. Membrane integrity and cell morphology were evaluated at 1 and 2 h respectively. No effect of pre-incubation with different NSAIDs on viability was observed (Figure 6A and B).

Different NSAIDs inhibit C5a-induced CD11b up-regulation while only ibuprofen blocks CXCL8-mediated effects on integrin CD11b

In our search for molecular mechanisms underlying the inhibitory effects of NSAIDs on neutrophil migration, we focused on integrin (CD11b) up-regulation. Treatments with different NSAIDs at 100 μM prevented C5a-induced CD11b up-regulation, assessed by flow cytometry analysis of both MFI (Figure 7A–C) and fluorescence intensity of molecules of equivalent soluble fluorochrome units (Figure 8A–C). Among the NSAIDs tested, only ibuprofen significantly reduced CXCL8-induced CD11b up-regulation, as assessed by both flow cytometry analysis methods (Figure 9A–C and Figure 10A–C).

Naproxen and oxaprozin block the PI3K/Akt phosphorylation pathway induced by C5a- and CXCL8

Another molecular pathway associated with neutrophil migration is represented by the phosphorylation of intracellular kinases (Montecucco *et al.*, 2008). Neutrophil migration towards C5a was significantly reduced by pretreatment of the cells with pharmacological inhibitors of the PI3K/Akt pathway (LY294002 and Akt inhibitor respectively) (Figure 11A). No effect was observed after pre-incubating cells with PD98059 (to inhibit MEK1/2, a kinase directly activating ERK1/2) or with SB203580 (to inhibit p38) (Figure 11A). As shown by Western blot (Figure 11B), incubation with C5a phosphorylated all the kinases investigated (Akt, ERK1/2, p38 MAPK). Among different NSAIDs, pre-incubation with naproxen or oxaprozin decreased C5a-induced phosphorylation of Akt (Figure 11B and C). These results indicated that neutrophil migration towards C5a was dependent on PI3K/Akt phosphorylation and that pre-incubation with naproxen or oxaprozin blocked C5a-mediated activation of this pathway.

In contrast, neutrophil migration towards CXCL8 was reduced by pretreatment with each of kinase inhibitors investigated (Figure 12A) and CXCL8 increased Akt, ERK1/2 and p38 MAPK phosphorylation (Figure 12B). Pre-incubation with naproxen or oxaprozin reduced CXCL8-induced phosphorylation of Akt (Figure 12B and C). In order to further confirm the selective action of naproxen and oxaprozin, but ibuprofen, on the PI3K/Akt pathway, we assessed the effects of different concentrations of NSAIDs on C5a- and CXCL8-induced Akt phosphorylation. As expected, despite a weak reduction in Akt phosphorylation, pre-incubation with different doses of ibuprofen (up to 100 μM) did not affect either C5a- or CXCL8-induced Akt phosphorylation (Figure 13A and B and Figure 14A and B), whereas pretreatment with the highest concentrations (50 and 100 μM) of naproxen or oxaprozin significantly inhibited both C5a- and CXCL8-induced Akt phosphorylation (Figure 13A and B and Figure 14A and B).

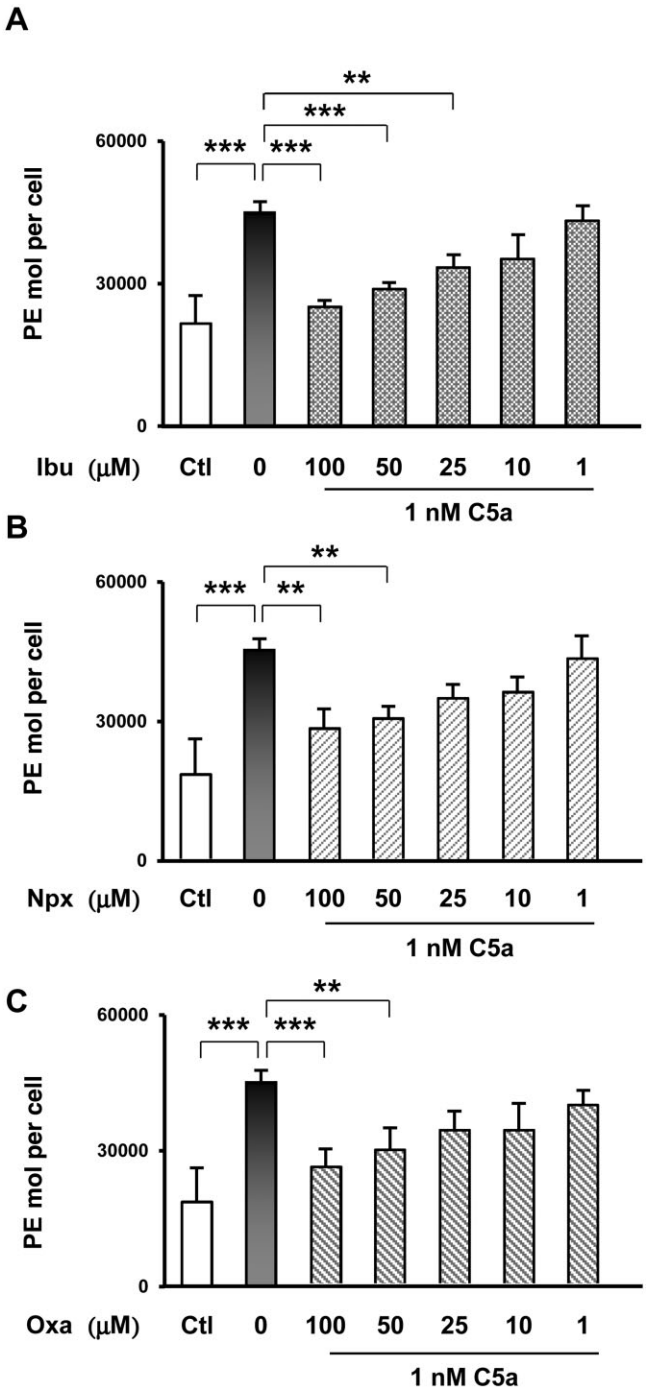

Figure 8

Analysis of CD11b expression (PE molecules per cell) on neutrophils pretreated with NSAIDs and stimulated with C5a. Flow cytometry analysis of PE-molecule expression on human neutrophils cultured for 1 h with or without different concentrations (up to 100 μM) of ibuprofen (Ibu) (A), naproxen (Npx) (B) or oxaprozin (Oxa) (C), and then stimulated with 1 nM C5a for 20 min. Data are expressed as mean ± SD of units of antibody bound per cell (PE mol per cell) of five experiments: $^{**}P \leq 0.01$;$^{***}P \leq 0.001$.

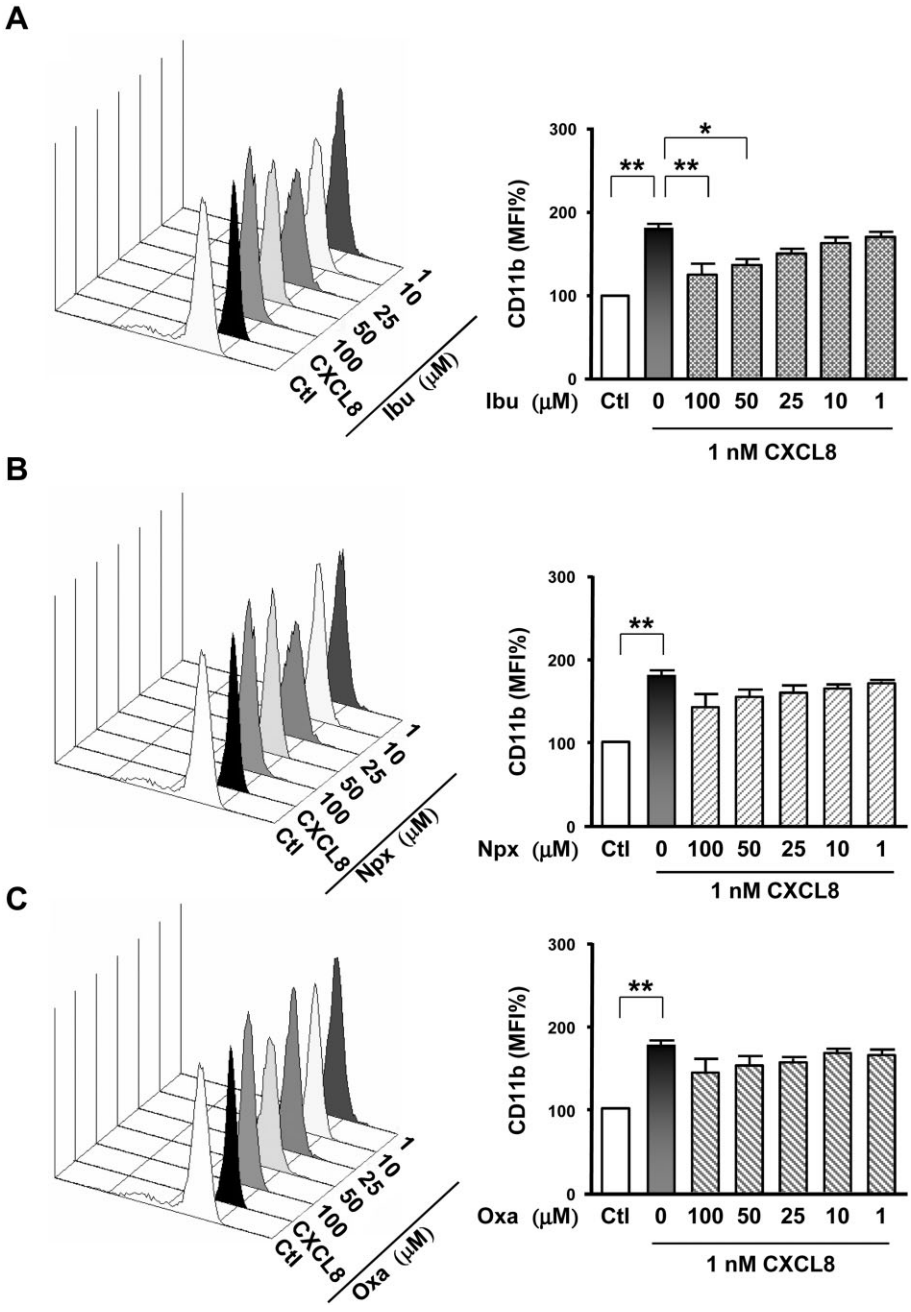

Figure 9

Analysis of CD11b expression (mean fluorescence intensity) on neutrophils pretreated with NSAIDs and stimulated with CXCL8. (A) Flow cytometry analysis of CD11b expression on human neutrophils cultured for 1 h with or without different concentrations (up to 100 μM) of ibuprofen (Ibu), naproxen (Npx) or oxaprozin (Oxa), and then stimulated with 1 nM CXCL8 for 20 min. (B) Quantification of the flow cytometry diagrams. Data are expressed as mean ± SD of percentages of MFI relative to the non-stimulated control (Ctl) of five experiments: $*P < 0.05$; $**P < 0.01$.

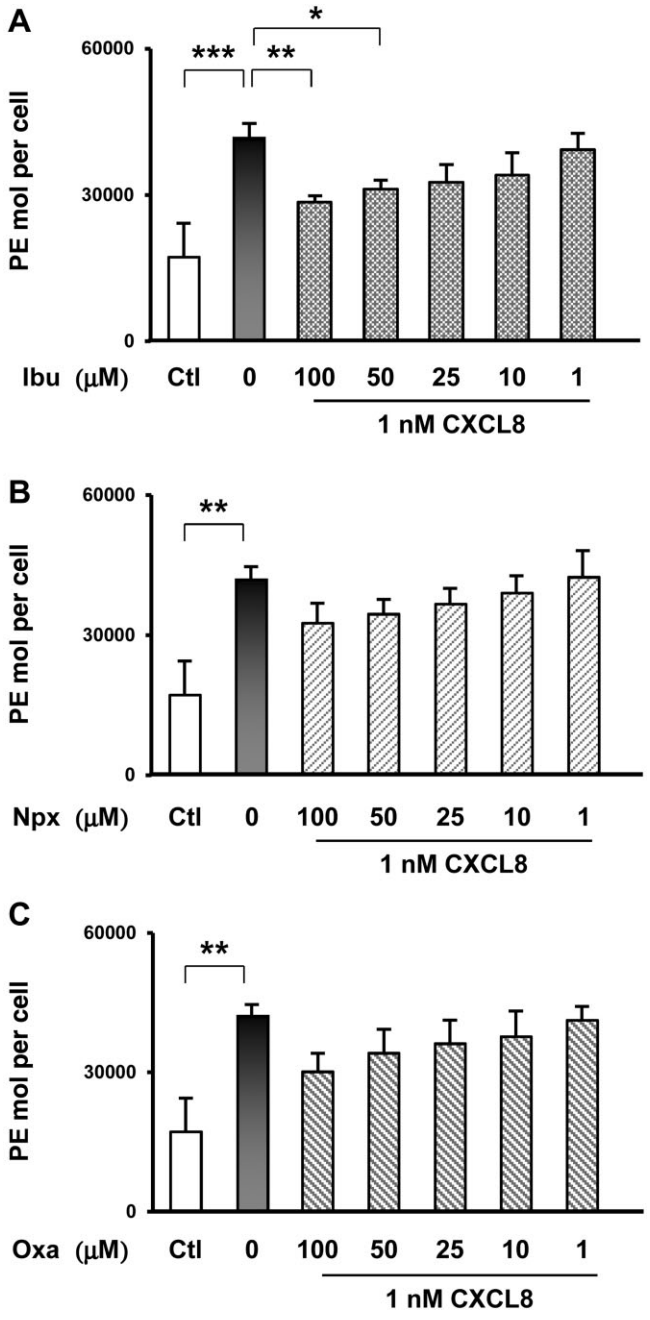

Figure 10

Analysis of CD11b expression (PE molecules per cell) on neutrophils pretreated with NSAIDs and stimulated with CXCL8. Flow cytometry analysis of PE-molecule expression on human neutrophils cultured for 1 h with or without different concentrations (up to 100 μM) of ibuprofen (Ibu) (A), naproxen (Npx) (B) or oxaprozin (Oxa) (C), and then stimulated with 1 nM CXCL8 for 20 min. Data are expressed as mean ± SD of units of antibody bound per cell (PE mol per cell) of five experiments: $^{**}P \leq 0.01$;$^{***}P \leq 0.001$.

NSAID-mediated effects on neutrophils were independent of COX activity and PGE_2 generation

In order to assess if NSAID-mediated effects on neutrophils were dependent on COX inhibition, we measured PG release in neutrophil cultures at the same incubation time points used in previous experiments. In contrast to the positive control PMA (Pouliot *et al.*, 1998), short-term incubation with C5a or CXCL8 did not affect PGE_2 release, with or without NSAIDs, compared with control medium (Figure 15A and B). In addition, to further exclude a potential effect of PGE_2 in our *in vitro* model, exogenous PGE_2, at a concentration (50 pg mL^{-1}) comparable to that detected in our cell cultures, was co-incubated with or without NSAIDs for 1 h before and during neutrophil migration towards control medium or chemoattractants. Incubation with exogenous PGE_2 did not affect NSAID-induced inhibition of neutrophil chemotaxis towards chemoattractants (Figure 16A and B). In contrast to results obtained in mice (Lemos *et al.*, 2009), pre-incubation with PGE_2 did not increase neutrophil migration towards chemoattractants (Figure 16A and B), suggesting that this mediator was not involved in the migration of human neutrophils in response to C5a and CXCL8, *in vitro*.

Discussion and conclusions

The anti-inflammatory effects of NSAIDs have been attributed to their inhibition of the enzymic activity of COX, which transforms arachidonic acid into PGs (Vane, 1971). These enzymes exist in two isoforms: COX-1 which is constitutively and ubiquitously expressed in human cells and COX-2, which is inducible particularly at inflammatory sites (Emery, 1996). Despite these beneficial properties, NSAIDs also induce anti-inflammatory effects (mainly on inflammatory cells), independently of the synthesis of PGs (Ottonello *et al.*, 2009). In this study, we focused on these secondary functions and, in particular, on NSAID-mediated inhibition of human neutrophil degranulation and recruitment in pro-inflammatory micro-environments, characteristic of chronic inflammatory diseases, such as rheumatoid arthritis (Khandpur *et al.*, 2013). The same pathophysiological concentration of 1 nM of C5a or CXCL8 (previously shown to both attract and activate neutrophils) (Haynes *et al.*, 2000; Montecucco *et al.*, 2008) was selected for all the experiments, with or without NSAIDs. In neutrophil granules, high quantities of MMPs and MPO are stored and rapidly released in response to high concentrations of C5a and CXCL8 (Chakrabarti and Patel, 2005; DiScipio *et al.*, 2006). Importantly, pretreatment with different NSAIDs did not influence C5a- or CXCL8-induced MPO degranulation. In contrast to other experimental models (Chakrabarti and Patel, 2005; DiScipio *et al.*, 2006), we did not show any significant increase in MMP-8 and MMP-9 degranulation in response to pathophysiological concentrations of C5a or CXCL8. Considering the lack of efficacy of NSAIDs on MPO degranulation, we believe that stimulation with very high concentrations (100 nM) of C5a and CXCL8 (Chakrabarti and Patel, 2005; DiScipio *et al.*, 2006) to further investigate the potential inhibitory activity of NSAIDs might be out of the scope of the present article.

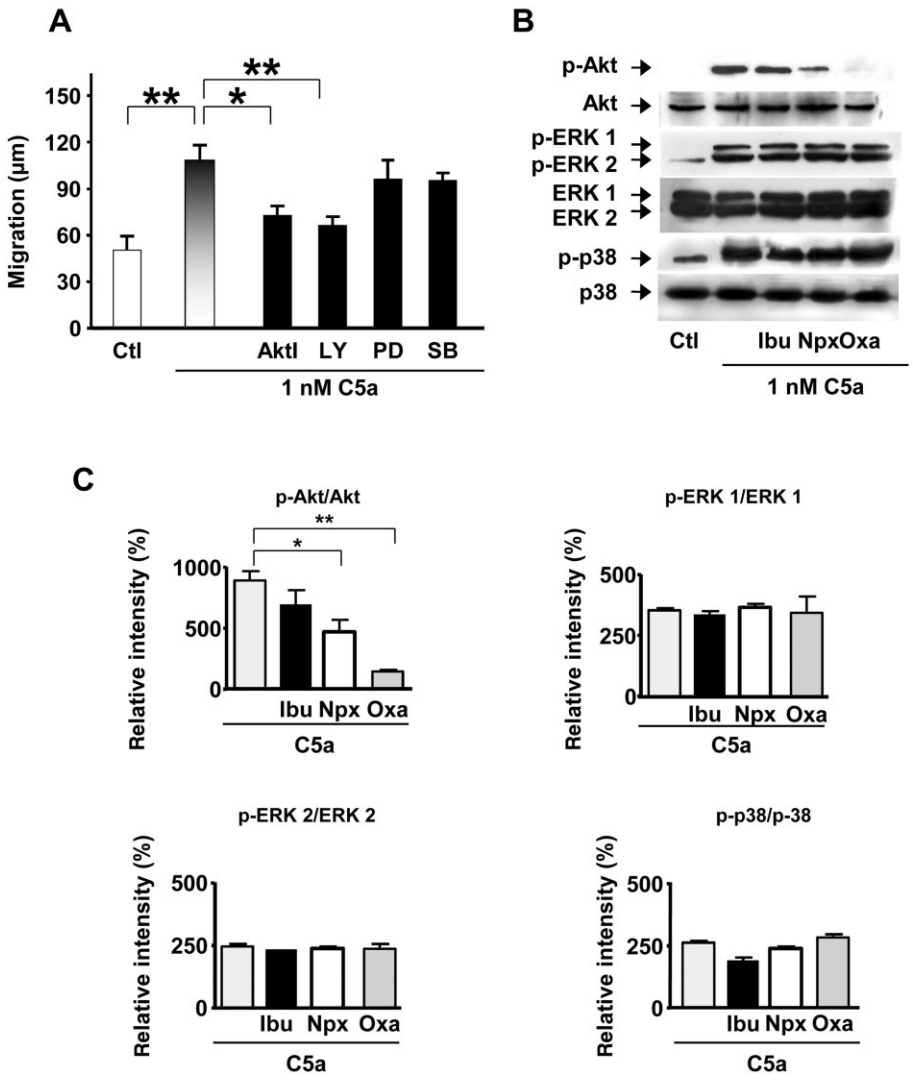

Figure 11

Oxaprozin and naproxen block PI3K/Akt phosphorylation induced by C5a. (A) Migration towards 1 nM C5a of human neutrophils pretreated in the absence (Ctl) or presence of 40 µM of Akt inhibitor (AktI), 10 µM PI3K inhibitor LY294002 (LY), 25 µM of ERK1/2 inhibitor PD98059 (PD) or 1 µM p38 MAPK inhibitor SB203580 (SB). Results are expressed as net migration and mean ± SD ($n = 5$), *$P < 0.05$;**$P < 0.01$; ***$P < 0.001$. (B) Representative Western blot analysis of Akt, ERK1/2 and p38 MAPK phosphorylation and total kinase protein; cells were pretreated for 1 h with medium alone (Ctl), 50 µM ibuprofen (Ibu), 50 µM naproxen (Npx) or 50 µM oxaprozin (Oxa), and then stimulated with or without 1 nM C5a for 5 min. (C) Densitometric analysis of phospho-proteins normalized to total amounts. Data are expressed as relative intensity, mean ± SEM ($n = 3$), **$P < 0.01$.

We focused on the potential inhibition by NSAIDs of C5a- and CXCL8-induced neutrophil chemotaxis. Both C5a and CXCL8 are considered as classical neutrophil chemoattractants and might potentially synergize in inflammatory microenvironments (Struyf *et al.*, 2005). The main finding of the present experiemtns is that a short-term pre-incubation (for 1h) with NSAIDs, concentration-dependently reduced neutrophil migration to C5a or CXCL8. These results confirm the pathophysiological relevance of a direct and potent anti-neutrophil effect of NSAIDs, independent of a delayed activity on PG synthesis (Locatelli *et al.*, 1993).

The migratory response of neutrophils involves cytoskeletal reorganization, formation and depolymerization of actin microfilaments (Van Haastert and Devreotes, 2004). NSAIDs potently inhibited these neutrophil functions *in vivo* at therapeutic plasma concentration (50–200 µg mL^{-1}) in rheumatoid arthritis patients (De Clerck *et al.*, 1997). Both C5a and CXCL8 are known to rapidly stimulate F-actin polymerization in human neutrophils (Westlin *et al.*, 1992). In our studies of migration, pretreatment with NSAIDs reduced F-actin polymerization in neutrophils, in response to C5a and CXCL8. Furthermore, the NSAIDs were used at a low thera-

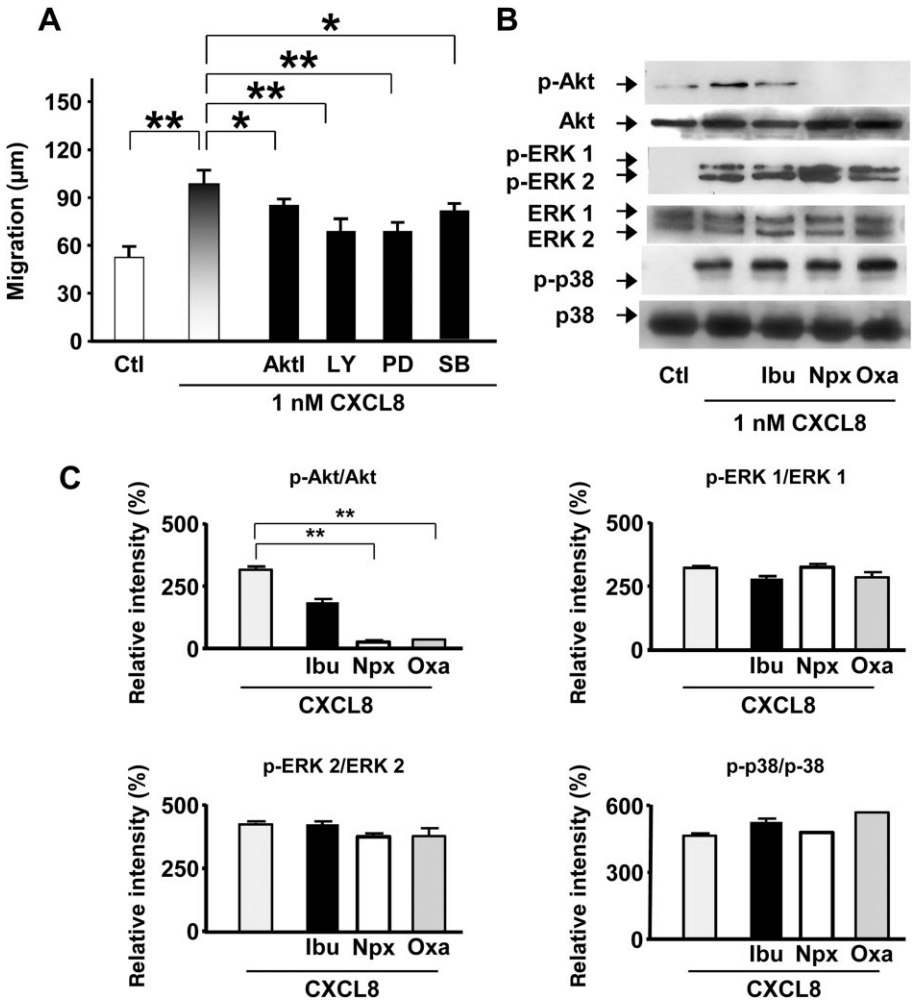

Figure 12

Oxaprozin and naproxen block PI3K/Akt phosphorylation induced by CXCL8. (A) Migration towards 1 nM CXCL8 of human neutrophils pretreated in the absence (Ctl) or presence of 40 µM of Akt inhibitor (AktI), 10 µM PI3K inhibitor LY294002 (LY), 25 µM of ERK1/2 inhibitor PD98059 (PD) or 1 µM p38 MAPK inhibitor SB203580 (SB). Results are expressed as net migration and mean ± SD ($n = 5$). *$P < 0.05$;**$P < 0.01$; ***$P < 0.001$. (B) Representative Western blot analysis of Akt, ERK1/2 and p38 MAPK phosphorylation, and total kinase proteins; cells were pretreated for 1 h with medium alone (Ctl), 50 µM ibuprofen (Ibu), 50 µM naproxen (Npx) or 50 µM oxaprozin (Oxa), and then stimulated without (Ctl) or with 1 nM CXCL8 for 5 min. (C) Densitometric analysis of phospho-proteins normalized to total amounts. Data are expressed as relative intensity, mean ± SEM ($n = 3$). **$P < 0.01$.

peutic dose (50 µmol L^{-1}, previously shown to inhibit COX-1 *in vitro*) (Kawai *et al.*, 1998; Furst *et al.*, 2005; Ottonello *et al.*, 2009), close to the circulating concentrations detectable in humans (34–58 µM for oxaprozin, 96 µM for ibuprofen, 260 µM for naproxen) (Lötsch *et al.*, 2001; Patel *et al.*, 2012). Importantly, there was no loss of viability in neutrophils exposed to these concentrations of NSAIDs, in all experiments performed.

In order to identify a potential molecular mechanism underlying NSAID-mediated effects, we focused on integrin up-regulation and intracellular kinase cascades (the MAPKs and Akt pathways) that have been recently identified as crucial transducers of the locomotory response in neutrophils (Ottonello *et al.*, 2005; Montecucco *et al.*, 2008; 2009b). The concentration–response curves using different NSAIDs showed a very limited inhibition by these compounds (only at the highest dose of 100 µM) on C5a-induced integrin up-regulation. On the other hand, a weak, but statistically significant, inhibition of CXCL8-induced integrin up-regulation was shown only with high concentrations (50–100 µM) of ibuprofen. Because we found significant inhibition of neutrophil chemotaxis with lower concentrations (i.e. 25–50 µM) of NSAIDs, it is difficult to correlate these effects on integrins with NSAID-induced inhibition of neutrophil chemotaxis towards C5a or CXCL8. Recent evidence also showed that neutrophil migration might result from a dynamic balance between ERK and p38 MAPK inhibition and activation, regulating the termination phase of

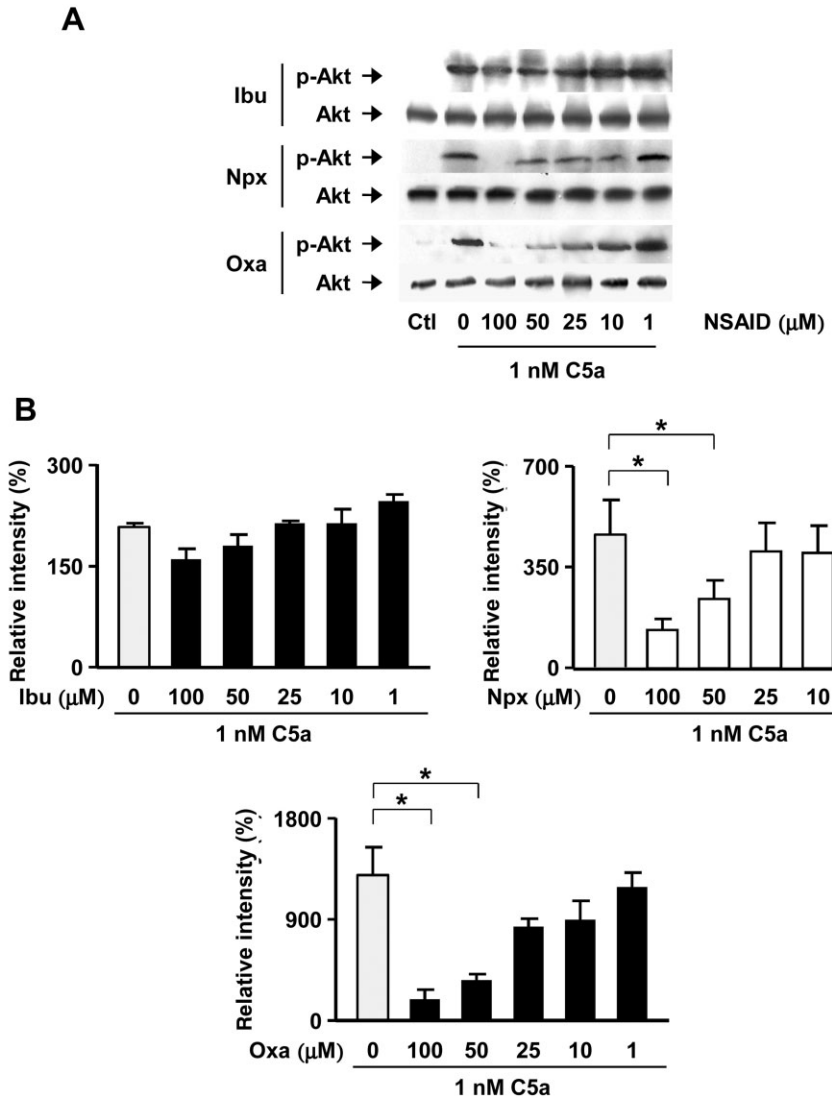

Figure 13

C5a-induced phosphorylation of PI3K/Akt was concentration-dependently inhibited by oxaprozin and naproxen. (A) Representative Western blots of Akt phosphorylation and total Akt protein; cells were pretreated for 1 h with medium alone (Ctl) or different doses (1–100 μM) of ibuprofen (Ibu), naproxen (Npx) or oxaprozin (Oxa), and then stimulated with or without 1 nM C5a for 5 min. (B) Densitometric analysis of phosphoproteins normalized to total protein amounts. Data are expressed as relative intensity, mean ± SEM (n = 3), *P < 0.05.

neutrophil locomotion (arrest) and preparing these cells to restart in order to reach the final destination (Liu *et al.*, 2012). These results have increased the complexity of kinase pathways underlying neutrophil migration and integrin up-regulation.

Pretreatment with naproxen and oxaprozin reduced both C5a and CXCL8-induced activation of the Akt pathway. Accordingly, pre-incubation with selective inhibitors of Akt reduced cell migration in response to both chemoattractants, indicating that this pathway was critical for neutrophil migration and that is was the molecular mechanism triggered by naproxen and oxaprozin. Evidence for anti-inflammatory properties mediated by oxaprozin via direct inhibition of Akt phosphorylation was recently shown in human monocytes (Montecucco *et al.*, 2009a; Ottonello *et al.*, 2009). However, it remains unclear if the NSAIDs inhibit this intracellular protein directly or if they affect another upstream target. On the other hand, the present study confirmed that NSAIDs could prevent neutrophil inflammation via pathways independent of COX inhibition and PGE generation. This conclusion was based on the lack of effect on PGE_2 release by NSAIDs in our cell cultures and the lack of activity of co-incubation with exogenous PGE_2 on the inhibitory effects of NSAIDs. In addition, no significant block of C5a- and CXCL8-induced intracellular pathways was induced by ibuprofen. This implies that the beneficial effects mediated by ibuprofen other intracellular pathways, possibly related to integrin down-regulation that could be associated with

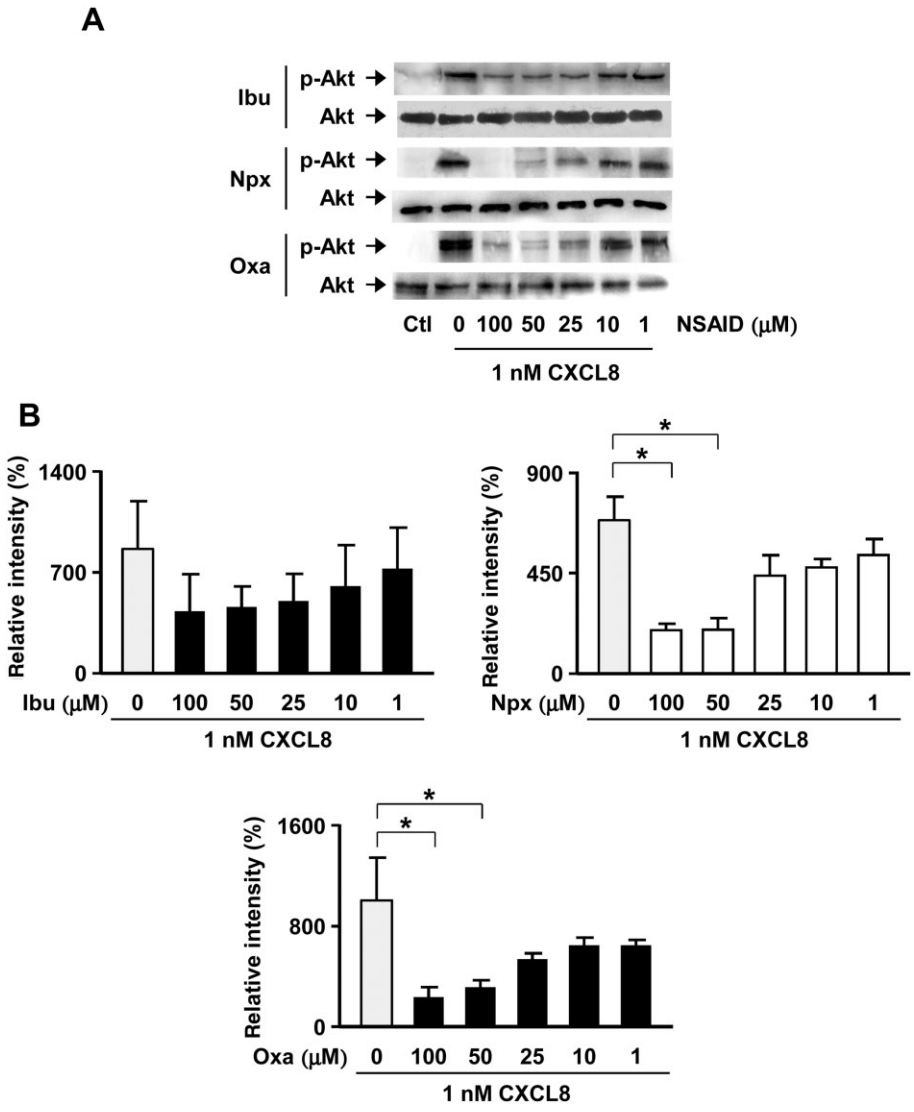

Figure 14

CXCL8-induced phosphorylation of PI3K/Akt was concentration-dependently inhibited by oxaprozin and naproxen. (A) Representative Western blots of Akt, phosphorylation and total Akt protein; cells were pretreated for 1 h with medium alone (Ctl) or different doses (1–100 μM) of ibuprofen (Ibu), naproxen (Npx) or oxaprozin (Oxa), and then stimulated without (Ctl) or with 1 nM CXCL8 for 5 min. (B) Densitometric analysis of phospho-proteins normalized to total amounts. Data are expressed as relative intensity, mean ± SEM (n = 3), *P < 0.05.

reduction of neutrophil migration (Montecucco *et al.*, 2009b). However, the intracellular mechanism by which ibuprofen blocked neutrophil migration in response to C5a and CXCL8 remains undefined and represents a major limitation of our study. Another potential weakness is that we only performed *in vitro* experiments. We chose this approach to focus on and clarify simple cellular and molecular mechanisms before planning any *in vivo* experiments. One advantage of our *in vitro* experiments was that we could use human primary cells rather than immortalized cell lines, and thus improve translation of our results into clinical conditions.

In conclusion, the present study has identified molecular and cellular mechanisms (independent of COX activity and PGE_2 release) underlying NSAID-mediated anti-inflammatory effects on neutrophil migration towards CXCL8 and C5a. Ibuprofen-mediated inhibition was associated with integrin down-regulation while naproxen and oxaprozin inhibited the PI3K/Akt pathway. These pathways might represent promising therapeutic targets to selectively block C5a- or CXCL8-mediated inflammatory responses in human neutrophils.

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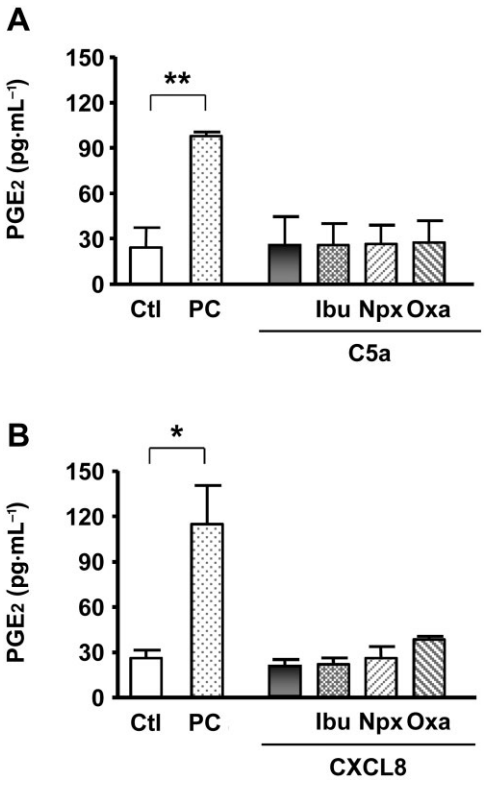

Figure 15

C5a or CXCL8 does not induce PGE_2 generation after short-term incubation *in vitro* in neutrophils. Cells were pretreated for 1 h with control medium (Ctl), 50 μM ibuprofen (Ibu), 50 μM naproxen (Npx) or 50 μM oxaprozin (Oxa). Then the cells were stimulated with or without 10 ng mL^{-1} PMA, as positive control (PC), 1 nM C5a (A) or 1 nM CXCL8 (B) for 30 min. The PGE_2 release in cell supernatants was tested. Data are expressed as mean ± SEM ($n = 3$). *$P < 0.05$; **$P < 0.01$.

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Author contributions

Maria Bertolotto, Franco Dallegri and Fabrizio Montecucco planned and performed the experiments, collected and analysed the results, and wrote and revised the manuscript. Paola Contini, Luciano Ottonello and Aldo Pende performed the experiments and revised the manuscript. All authors accepted this submitted version of the article.

Conflict of interest

None.

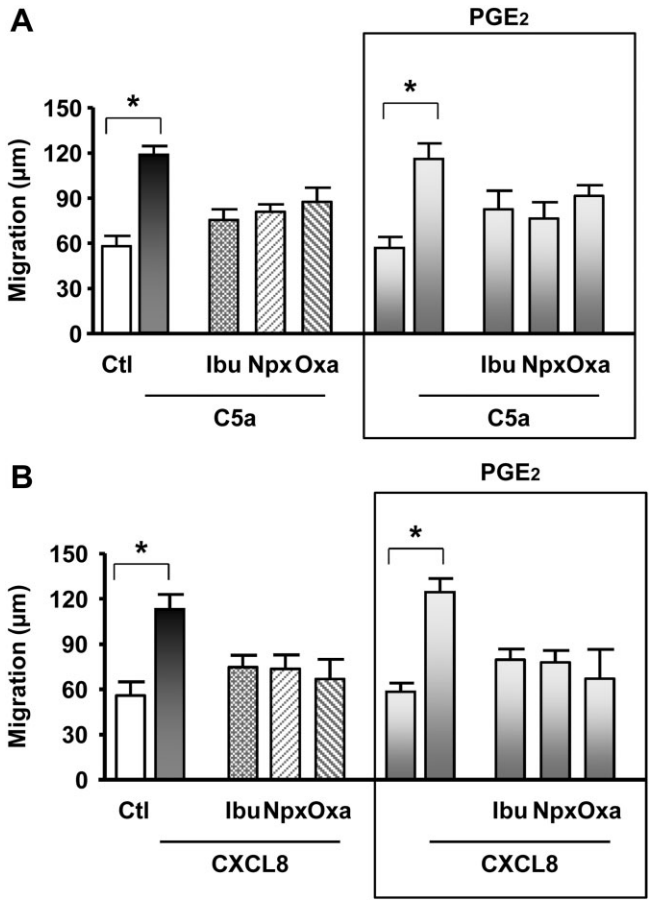

Figure 16

Co-incubation with PGE_2 does not affect neutrophil migration. Neutrophil migration towards control medium (Ctl), 1 nM C5a (A) or 1 nM CXCL8 (B) of neutrophils pretreated for 1 h with or without 50 μM ibuprofen (Ibu), 50 μM naproxen (Npx) or 50 μM oxaprozin (Oxa), and concomitant 50 pg mL^{-1} PGE_2. Results are expressed as migration (μm) towards control medium (Ctl) or chemoattractants and as mean ± SD of three experiments. *$P < 0.05$.

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