Antiallergic Cromones Inhibit Neutrophil Recruitment Onto Vascular Endothelium via Annexin-A1 Mobilization

Samia Yazid, Giovanna Leoni, Stephen J. Getting, Dianne Cooper, Egle Solito, Mauro Perretti, Roderick J. Flower

Objective—To determine whether the inhibitory action of the antiallergic cromone “mast cell stabilizing” drugs on polymorphonuclear leukocyte (PMN) trafficking is mediated through an annexin-A1 (Anx-A1) dependent mechanism.

Methods and Results—Intravital microscopy was used to monitor the actions of cromones in the inflamed microcirculation. Reperfusion injury provoked a dramatic increase in adherent and emigrated leukocytes in the mesenteric vascular bed, associated with augmented tissue levels of myeloperoxidase. Nedocromil, 2 to 20 mg/kg, significantly \((P<0.05)\) inhibited cell adhesion and emigration, as well as myeloperoxidase release, in wild-type but not Anx-A1 \(^{-/-}\) mice. Short pretreatment of human PMNs with nedocromil, 10 nmol/L, inhibited cell adhesion \((P<0.05)\) in the flow chamber assay, and this effect was reversed by specific anti-AnxA1 or a combination of antiformyl peptide receptors 1 and 2, but not irrelevant control, antibodies. Western blotting experiments revealed that cromones stimulate protein kinase C-dependent phosphorylation and release Anx-A1 in human PMNs.

Conclusion—We propose a novel mechanism to explain the antiinflammatory actions of cromones on PMN trafficking, an effect that has long puzzled investigators. (Arterioscler Thromb Vasc Biol. 2010;30:1718-1724.)

Key Words: nedocromil cromoglycate PKC inflammation ■ FPR receptors ■ reperfusion injury ■ vascular biology

Cromones are a group of antiallergic drugs; sodium cromoglycate and sodium nedocromil are exemplars. Early studies on the mechanism of action of these mast cell stabilizers suggested that they blocked mast cell degranulation and, thus, mediator release, when this was triggered by various stimuli, including IgE.\(^1\) However, these drugs can affect other facets of the inflammatory process, including inhibition of eicosanoids\(^2\) and cytokine release.\(^3\)

Leukocyte emigration\(^4\) is also inhibited by these drugs eg, cromoglycate and ketotifen exhibit a protective role in neutrophil-dependent pathological features, including the intestinal\(^5\) and pulmonary\(^6\) ischemia-reperfusion (IR) model. Mast cells, located in close apposition to the vessels, are an important source of proinflammatory mediators released at the site of inflammation.\(^7\) Mast-cell derived mediators activate adhesion molecules from the local environment, leading to leukocyte recruitment,\(^7\) using a well-characterized multistep mechanism.\(^8\) Clearly, inhibition of the release of mediators from these mast cells could explain the effect of the cromones on polymorphonuclear leukocyte (PMN) trafficking. However, other research\(^9\) points to a separate and direct effect of these compounds on PMNs.

Annexin-A1 (Anx-A1) is a 37-kDa glucocorticoid (GC)–regulated protein that mimics the effects of proteins in several in vivo and in vitro model systems.\(^10-12\) GCs induce the Anx-A1 gene in many cells and also increase secretion of the protein from existing intracellular pools by stimulating protein kinase C (PKC) activity.\(^13\) Once secreted, the protein acts in a paracrine/autocrine fashion, using formyl peptide receptors (FPRs)\(^14,15\) to produce its biological effects. The N-terminal region of Anx-A1 bears the biological activity of the protein, and phosphorylation on the serine\(^27\) residue is crucial for protein export and secretion.\(^13\) While studying the rapid mechanism of action of GC in a promonocytic U937 cell line, we noted that cromones promoted the rapid release of Anx-A1.\(^16\) This occurred through inhibition of phosphatase PP2A, which constitutively limits PKC activity, thereby potentiating the phosphorylation and release of Anx-A1.\(^16\)

Anx-A1 is found in high amounts in circulating neutrophils and monocytes,\(^17\) from which the protein is externalized and cleaved after leukocyte adhesion to the endothelium in vitro.\(^18\) The protein strongly inhibits neutrophil recruitment in vivo\(^19\) and in vitro.\(^20,21\) In this study, we sought to investigate the mechanism of action of cromones on PMN emigration.

Methods

Two distinct, yet complementary, models were used. In an in vivo reperfusion injury model using Anx-A1 \(^{-/-}\) and an in vitro flow...
chamber assay using human cells together with a specific neutralizing anti–Anx-A1 antibody, we assessed the effect of the cromones on the internalization of FPR1 and FPR2 by fluorescence-activated cell sorter analysis and flow chamber assay. Cromones were also tested in zymosan-induced peritonitis, an acute inflammation model. A full description of the methods used is available in the supplemental materials (available online at http://atvb.ahajournals.org).

**Results**

**Alterations in the Mouse Mesenteric Microcirculation After IR and the Effect of Nedocromil**

In this model of IR in the mesenteric circulation, clamping and reopening of the superior mesenteric artery induced a significant increase in the number of adherent and emigrated cells observed microscopically in wild-type (WT) mice, when compared with the sham operated on animals (Figure 1A). This activation of the inflammatory process within the postcapillary venule endothelium is attenuated by the administration of nedocromil, 2 mg/kg, given 45 minutes before reperfusion.

Then, we investigated the mechanism by which nedocromil exerts this protective effect. The IR protocol induced a reduction in PMN rolling velocity in μm/s (V<sub>WBC</sub>) (rolling velocity) in WT mice, which was associated with a significant increase in the degree of cell adhesion and emigration, as assessed 45 minutes after reperfusion (Figure 1B). Treatment of mice with a low dose of nedocromil, 2 mg/kg, strongly inhibited cell adhesion and emigration but had no effect on V<sub>WBC</sub>. At the higher dose of 20 mg/kg, nedocromil inhibited all 3 parameters under observation (ie, cell rolling, velocity, and adhesion and emigration), although the latter did not reach statistical significance (Figure 1B).

Next, we tested the potential involvement of Anx-A1 in the observed intravascular effects of nedocromil using Anx-A1<sup>−/−</sup> mice. In these animals, the IR procedure did not alter V<sub>WBC</sub> but produced the expected increase in cell adhesion and emigration. Within the time frame of these experiments, no difference between the genotypes was observed with respect to the cellular response in the microcirculation (Figure 1B). However, nedocromil, 2 or 20 mg/kg, was without any discernable effect on the leukocyte-endothelium interactions promoted by the IR procedure in Anx-A1<sup>−/−</sup> mice. These in vivo data strongly suggest that nedocromil exerts its protective effects in the inflamed microcirculation through the antiinflammatory protein Anx-A1. Hemodynamic parameters were also measured. The administration of nedocromil, 2 or 20 mg/kg, significantly increased cell flux and wall shear rate in the WT mouse relative to PBS treatment (supplemental Table I), but the compound did not alter the hemodynamic parameters in Anx-A1<sup>−/−</sup> mice (supplemental Table II).

**Measurement of Myeloperoxidase in the Mesenteric Tissue**

PMN accumulation into the mesenteric tissue was also assessed by quantifying deposition of myeloperoxidase (MPO) by infiltrated cells (all data shown as mean±SE). Mesenteric tissue samples from WT and Anx-A1<sup>−/−</sup> mice that had been subjected to IR exhibited a significant increase in MPO: 0.26±0.04 to 1.06±0.08 U/mg and 0.49±0.02 to 1.58±0.23 U/mg for WT and Anx-A1<sup>−/−</sup> mice, respectively (Figure 1C). Mesenteric tissue samples from WT mice that had been treated with nedocromil, 2 mg/kg, had significantly less MPO activity (0.56±0.06 U/mg); more important, the inhibitory effect of nedocromil was lost (1.64±0.19 U/mg) in the Anx-A1<sup>−/−</sup> mice. Anx-A1<sup>−/−</sup> mouse tissue displayed a trend to higher MPO values versus WT tissue samples (Figure 1C).
Effects of Nedocromil on Human Neutrophil-Endothelium Interaction

Human umbilical vein endothelial cells (HUVECs), stimulated with tumor necrosis factor (TNF)/H9251 for 4 hours (a point that allows sufficient time for de novo synthesis of adhesion molecules20), were used in this assay. Activation of HUVEC monolayers provoked a marked capture of PMNs, and both rolling and adherent leukocytes could be visualized and counted off-line. Although nedocromil, 10 nmol/L, preincubated for 5 minutes with the PMNs, had no effect on the number of leukocytes rolling or captured on the endothelium, the compound significantly decreased (P<0.05) the number of cells adherent on the endothelium (n=3, P<0.05) (Figure 2A).

To ascertain the role of Anx-A1 in the mechanism of action of nedocromil, the effect of a specific neutralizing anti–Anx-A1 antibody was tested. PMNs were preincubated for 20 minutes with 10-μg/mL neutralizing Anx-A1 or irrelevant isotype-matched monoclonal antibodies and then incubated for 5 minutes with the PMNs, had no effect on the number of leukocytes rolling or captured on the endothelium, the compound significantly decreased (>50%) the number of cells adherent on the endothelium (n=3, P<0.05) (Figure 2A).

Because Anx-A1 exerts its antiinflammatory effects through the activation of FPRs, we assayed to the effect of highly specific blocking anti–FPR122 and anti–FPR220 antibodies, 5 μg/mL, 1 hour before treatment (Figure 2B). Interestingly, the antiadhesive effect of nedocromil sodium was partially abrogated by either antibody, but the combination of both could totally block the nedocromil inhibitory adhesive effect of the PMNs on the monolayer. The nonneutralizing monoclonal antibody was without any effects in this system (data not shown).

Effect of Nedocromil on FPR Family Receptors

To provide additional support for our notion that Anx-A1 acts through FPR receptors in this flow chamber system, the expression of FPR-1 and FPR-2 receptors on the PMN cell surface was analyzed by a fluorescence-activated cell sorter after treatment with cromones. Figure 3 indicates that nedocromil rapidly (within 5 minutes) causes internalization of both receptors (15% to 20%). Unrelated antiinflammatory drugs were assessed as positive controls.

Collectively, these in vivo and in vitro data support our hypothesis that cromones can exert their anti-PMN effects by releasing endogenous Anx-A1. Thus, the effect of these drugs on Anx-A1 disposition in human PMNs was further investigated.

Effect of Cromones on Anx-A1 Phosphorylation and Secretion by Human PMNs

To address our next aim, the phosphorylation of PKC and Anx-A1 in cell lysates of PMNs pretreated with different drugs was determined. Western blotting analysis reveals the rapid phosphorylation of PKC in human PMN cell lysates after 5 minutes of treatment with either nedocromil, 10 nmol/L; cromoglycate, 10 nmol/L; or ketotifen, 10 nmol/L (Figure 4A). This is accompanied by an increase in phosphorylated serine 27 Anx-A1 which is essential for the secretion of the protein.13,16 No changes in total amount of Anx-A1 could be detected in the PMN extracts (Figure 4A and B). The same phosphorylation pattern of the protein was observed when PMNs were treated with either dexamethasone, 2 nmol/L, or okadaic acid, 10 nmol/L.

The increments in phosphorylated serine 27 Anx-A1 would be indicative of protein mobilization and secretion, according to our model. Next, we monitored cell-surface
Anx-A1 in human PMNs, using flow cytometry analysis. Under basal conditions, few PMNs (<3%) displayed Anx-A1 on their plasma membrane (as detected with a highly specific monoclonal antibody) (Figure 5A and B), whereas pretreatment with ketotifen, 10 nmol/L, or dexamethasone, 2 nmol/L, significantly increased the proportion of cells positive for Anx-A1 (Figure 5B), with cromoglycate, nedocromil, and okadaic acid being somewhat less active in this assay (Figure 5B).

The final step of Anx-A1 mobilization in activated PMNs is secretion in the medium. Thus, next we assessed, using a validated ELISA,23 whether we could detect release of Anx-A1 into the extracellular milieu after cromone treatment. Nedocromil, cromoglycate, nedocromil, and okadaic acid were more effective inducers of Anx-A1 release into the medium than either ketotifen or dexamethasone (Figure 5C). These observations could explain, in part, the apparent discrepancies observed between the drug effects in the static adhesion assay, presented in supplemental Figure I.

Effect of Cromones in an Acute Inflammation Model

Finally, we tested whether the effects of cromones on PMN-endothelium interaction could be translated to attenuation of PMN infiltration in a model of acute peritonitis. The marked (>10 million cells) PMN accumulation promoted by zymosan was susceptible to inhibition by 2 unrelated anti-inflammatory drugs, the melanocortin analogue melanotan II (MTII) and indomethacin (a cyclo-oxygenase inhibitor) (Figure 6A). Because neither of these drugs act through an Anx-A1-dependent pathway, they displayed similar inhibition of cell influx in both WT and Anx-A1−/− mice (MTII, 26% and 33%, respectively; and indomethacin, 80% and 83%, respectively).

Next, nedocromil was tested in this model. Probably because of poor pharmacokinetics, the doses required to inhibit PMN migration in this model are higher than anticipated from the in vitro data, with doses less than 10 mg/kg being without effect (Figure 6B). More important, as in the case of dexamethasone,24 nedocromil acts in a genotype-dependent manner, with doses ranging from 10 to 50 mg/kg, producing a graded inhibition between 5% and 49% in WT mice. No consistent effect was observed in Anx-A1−/− mice. Cromoglycate produced a comparable inhibitory profile, although doses of 50 to 200 mg/kg were necessary to inhibit PMN migration in this model (data not shown).

Discussion

There is compelling evidence supporting the notion that mast cells regulate leukocyte influx in disease. Indeed, the efficacy
of “mast cell–stabilizing drugs” and the phenotype of mast cell–deficient mice support this concept in lung inflammation,25 gut inflammation,26 blood-brain barrier permeability,27 plasma extravasation,28 and intestinal inflammation.29 Other studies show how leukocyte influx can also occur in a mast cell–independent manner9 and how mast cell stabilizers may act on inflammatory cells other than the mast cell, although a mechanism that could explain such antiinflammatory effects has never been offered. Our study indicates Anx-A1 as a major molecular target for these effects of the cromones.

Recently, it was reported that Anx-A1, a GC-regulated antiinflammatory protein, is secreted by antiallergic drugs when cells have first been “primed” with low GC doses.16 This was proposed to occur secondary to PKC activation (triggered by GCs), which is necessary to initiate Anx-A1 phosphorylation; the cromones potentiated this effect by inhibiting a phosphatase (probably PP2A), which limits PKC activation and activity at the plasma membrane. Therefore, the effect on Anx-A1 phosphorylation and secretion by PMNs, of a well-characterized PP2A inhibitor, such as okadaic acid, was also assessed. Indeed, in line with a previous study using the U937 promonocytic cell line, a correlation between the effects of cromones and okadaic acid on Anx-A1 distribution in the PMNs was observed. Thus, the model whereby 2 processes are required for Anx-A1 secretion, phosphorylation as a prerequisite for externalization is not limited to the mast cell and monocyte; it is also valid for human PMNs and, thus, likely to be a paradigm in the biology of this protein.

We have not addressed the actual mechanism of Anx-A1 secretion from the PMNs in this study. These cells contain large amounts of the protein (≤4% of total intracellular proteins30); the intracellular distribution is complex,31 with much (approximately 50% to 60%) of Anx-A1 being located in gelatinase granules,32 which are externalized, along with their contents, after adherence to endothelial cells. After this, Anx-A1 is eventually cleaved by the protease PR3 as well as other proteases.33 Depending on the mechanism of PMN activation, Anx-A1 may also be released when attached to microparticles.34 It is not clear whether it is the gelatinase pool or the cytosolic Anx-A1 pool (not in granules or vesicles) that is the target for PKC in the human PMNs; however, in our experiments (supplemental Figure II) using markers to identify the different granule subsets in PMNs, it seemed that the latter pool of Anx-A1 to be mobilized after PKC-mediated phosphorylation.

Because Anx-A1 produces antiinflammatory effects through potent inhibition of the leukocyte migration process,
we hypothesized that the functional release of the protein by cromones could not only explain their inhibition of eosinoid release but also underlie their inhibitory effects on cell influx in acute and chronic inflammation. Mast cell activation produces a sustained leukocyte influx through release of mediators, such as histamine. This can occur in an IgE-dependent, and independent, manner. One example of the latter is IR injury, in which mast cells are crucial regulators of leukocyte influx. Therefore, in the present study, we have used the mouse mesenteric microcirculation inflamed with an IR procedure and monitored (by intravital microscopy) the process of leukocyte recruitment onto the postcapillary venule endothelium. In line with previous studies conducted in the rat using cromoglycate or ketotifen, nedocromil induced a decrease in leukocyte adhesion and emigration. This was absent in our Anx-A1−/− mouse colony, providing strong evidence for an important role for this effector of endogenous antiinflammatory regulation. Conscious that Anx-A1−/− mice exhibit a tendency to exacerbate inflammatory responses, we have also tested a supramaximal dose of nedocromil to ensure that its effect was not masked by the more pronounced inflammatory response seen in these knockout mice. No difference between WT and Anx-A1−/− mice could be demonstrated in this experimental setting, indicating that the endogenous protein is not operative within the time frame used in these experiments. In fact, we know that Anx-A1−/− mice display augmented cellular responses in the mesenteries after IR at later points (>6 hours) (unpublished data).

Moreover, using nedocromil as a prototype, we could assess the antiinflammatory properties of cromones in a model in which intense PMN accumulation occurs. In this experiment, significant inhibition and, more important, marked reliance on endogenous Anx-A1 were observed. The effect of nedocromil was lost in Anx-A1−/− mice, in line with that reported for dexamethasone: historic data from our laboratory show that, given at 1 mg/kg, this steroid provokes acute Anx-A1–dependent inhibition, reaching approximately 80% inhibition in WT, but only 9% inhibition in Anx-A1−/− mice. As expected, the assay can discriminate the contribution of endogenous Anx-A1 because a melanocortin receptor agonist and indomethacin provoked similar degrees of inhibition of PMN accumulation in both WT and Anx-A1−/− mice.

These actions of the cromones can be compared with those obtained with dexamethasone, used as a positive control because it also relays, in part, on Anx-A1 mobilization for its antileukocytic effects. Additional evidence that this mechanism is relevant to the antiinflammatory properties of cromones comes from our experiments using a specific neutralizing anti–Anx-A1 antibody in a flow chamber model. Human PMN-endothelial monolayer interactions were monitored under defined flow conditions using a human system in vitro that physiologically resembles the events occurring in the microcirculation during inflammation. HUVECs were stimulated with tumor necrosis factor α for 4 hours to promote human PMN adherence. Although nedocromil was without any effect on the capture and rolling of the PMNs onto the HUVECs, a significant decrease in the number of

PMNs adherent to the monolayers was observed: this inhibition was abolished when the PMNs were pretreated with a specific anti–Anx-A1–neutralizing antibody. This suggested that the antimigratory effect of nedocromil on neutrophil recruitment is an Anx-A1–mediated event.

This study mainly addressed the mechanism by which the cromones exert their inhibitory effect on leukocyte recruitment (and used protocols to focus on PMNs as the main target); we have not investigated their effect on other molecules implicated in the trafficking cascade. Cromoglycate can downregulate vascular CD54 (or intercellular adhesion molecule-1) expression in a rat lung IR model on endothelial cells interacting with peripheral blood mononuclear cells of asthmatic patients or in biopsy findings of their bronchial mucosa. These effects could contribute to the cromone inhibitory effects seen on leukocyte recruitment in the IR model because increased levels of vascular intercellular adhesion molecule-1 are to be anticipated. However, in the flow chamber assay, tumor necrosis factor stimulation of HUVECs for 4 hours increases E-selectin (CD62E) but has little effect on CD54 expression, which does not peak until 16 hours of stimulation. Moreover, it is unlikely to be working via any targets on the endothelium because the drugs were added to the neutrophils before the flow assay.

In conclusion, we have established that cromones can act directly on human and mouse PMNs to regulate their recruitment on activated endothelial monolayers or postcapillary venule endothelium, respectively. To our knowledge, we also provide the first evidence, in vivo and in vitro, that these effects require the endogenous antiinflammatory protein Anx-A1, showing (in human PMNs) externalization and release of this protein. These findings could explain some observations originally made by R.E.C. Altounyan, who speculated that cromones might be endowed with clinical properties similar to corticosteroids. In addition to elucidating a mechanism through which cromones exert antiinflammatory properties, our study also suggests further uses for these drugs in the context of pathological features driven by excessive PMN activation examples being vasculitis and gouty arthritis.

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Disclosures

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Supplement Material

Animal work was performed according to Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986) and was approved by the Queen Mary University of London Ethics Committee (London, United Kingdom). Human cells were prepared according to a protocol approved by the East London & the City Local Research Ethics Committee (no. 06/Q605/40; P/00/029 ELCHA, London, United Kingdom).

Model of acute inflammation: zymosan peritonitis

Briefly, mice were injected i.p. with 1mg of zymosan A (Sigma-Aldrich, Poole, UK) diluted in 0.5ml phosphate buffered saline. Animal were sacrificed after 4 h later by carbon-dioxide exposure and peritoneal cavities were lavaged with 3ml PBS containing 3mM EDTA and stained in Turk’s solution (0.01% crytal violet in 3% acetic acid). Total and differential cell counts were made using an Olympus B061 microscope (Olympus, London, UK). Nedocromil sodium and cromoglycate sodium were administered s.c. 1h prior to zymosan injection. MTII (Ac-Nle⁴-c[Asp⁵, D-Phe⁷-Lys¹⁰]NH₂; Bachem, Saffron Walden, UK), an anti-inflammatory melanocortin receptor agonist melanotan II, and indomethacin, a cyclo-oxygenase inhibitor, were used as controls as they work through Anx-A1 independent mechanisms.

Intravital Microscopy of the mouse mesentery.

Male mice (~20 g body weight) were maintained on a standard chow pellet diet and had free access to water, with a 12h light/dark cycles. The Anx-A1 null and wild type animals (strain C57BL/6; B&K Hull, UK) were used 7 days after arrival. Intravital microscopy was performed. Mice were anesthetized intraperitoneally (i.p) with a mixture of 7.5 mg/kg xylazine and 150 mg/kg ketamine hydrochloride. Animals were placed in supine position on a heating pad (37°C) for maintenance of body temperature. A polyethylene catheter (PE-10 with an internal
diameter of 0.28 mm) was placed into the internal jugular vein for administration of drugs. Mesenteric ischaemia was induced with a micro-aneurysm clip (Harvard Apparatus; Kent, UK), clamping the superior mesenteric artery for 30 min. The clip was then removed, and reperfusion allowed for 45 min for evaluation of white blood cell reactivity. Sham-operated animals underwent the same surgical procedure but the superior mesenteric artery was not clamped.

The mesenteric vascular bed was exteriorized, placed on a viewing Plexiglas stage, and mounted on a Zeiss Axioskop “FS” with a water-immersion objective lens (magnification x 40; Carl Zeiss, Welwyn Garden City, UK). Tissue preparations were transilluminated with a 12 V, 100W halogen light source. A Hitachi charge-coupled device colour camera (model KPC571; Tokyo, Japan) acquired images that were displayed onto a Sony Trinitron colour video monitor (model PVM 1440QM; Tokyo, Japan) and recorded on a Sony super-VHS videocassette recorder (model SVO-9500 MDP; Tokyo, Japan) for subsequent offline analysis. Mesenteries were superfused with thermostated (37ºC) bicarbonate-buffer solution (g/l: NaCl 7.71; KCl 0.25; MgSO₄, 0.14; NaHCO₃ 1.51; and CaCl₂, 0.22, pH 7.4, gassed with 5% CO₂/95% N₂) at a rate of 2mL/min. After positioning the microcirculation under the microscope, a 5 min equilibration period preceded recording for quantitative measurements. Analyses of leukocyte-endothelium interactions were made in 3 to 4 randomly selected post-capillary venules (diameter between 20 to 40 µm; visible length of at least 100 µm) for each mouse.

Quantification of microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. White blood cell rolling velocity (V_{WBC}) was determined from the time required for a leukocyte to roll a given distance along the length of the venule, and is reported in µm s⁻¹. Rolling leukocyte flux was determined by counting the number of
leukocytes passing a reference point in the venule per min and expressed as cells per minute (cell min\(^{-1}\)). Leukocyte adhesion was measured by counting cells that remained stationary for 30s or longer within a 100µm section of vessel. Leukocyte emigration from the microcirculation into the tissue was calculated by counting the number of cells in a 100x50 µm\(^2\) area on both sides of the 100 µm vessel segment. Red blood cell centerline velocity was measured in venules with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, Dallas, USA) and venular wall shear rate was determined based on the Newtonian definition: wall shear rate = 8,000 [(red blood cell velocity/1.6)/venular diameter].

In some cases, nedocromil was given at the dose of 20mg/kg i.v. (either in WT or Anx-A1\(^{-/-}\) mice) at the end of the ischaemic phase (30min), immediately prior to reperfusion.

**Measurement of myeloperoxidase (MPO) activity**

Leukocyte MPO activity was assessed by measuring the H\(_2\)O\(_2\) dependent oxidation of 3,3',5,5'-tetramethylbenzidine (TMB). At the end of the intravital microscopy procedure, mesentery tissues were harvested and stored at -80°C. Mesenteric tissue fragments from sham-operated animals and mice subjected to IR were homogenized in PBS containing 0.5% hexadacyl trimethylammonium bromide detergent. The homogenate was centrifuged at 13,000xg for 5 min prior to adding 20-µl supernatant volumes to 160 µl of tetramethylbenzidine and 20µl of H\(_2\)O\(_2\) in 96-well plates. Plates were incubated for 5 min at room temperature and optical density was read at 620nm using GENios (TECAN, Reading, UK). Assays were performed in duplicate and normalized for protein content (BCA protein assay, Pierce, UK).
**Flow chamber assay**

Human umbilical vein endothelial cells (HUVECs) were isolated within our laboratory from fresh umbilical cords kindly supplied by the midwifery staff of the Maternity Unit, Royal London Hospital, Whitechapel, UK. Confluent HUVEC monolayers were stimulated with TNF-α (10ng/mL; Sigma-Aldrich, Poole, UK) for 4 h. Following the informed consent, alluded above, peripheral venous blood was collected from healthy volunteers with a 21-gauge needle and transferred to a 50mL Falcon tube containing 1/10 volume of 3.2% sodium citrate. Blood was first diluted 1:1 in 1640 RPMI medium before separation in a double density gradient. After isolation and washing of the PMNs, contaminating red blood cells were removed by hypotonic lysis. PMNs were diluted to 1x10⁶/mL in Dulbecco phosphate-saline (DPBS) supplemented with Ca²⁺ and Mg²⁺, and incubated with or without nedocromil sodium (10nM) prior to flow for 5 min at 37°C. In some experiments, PMNs were either pre-incubated for 30 min at 37°C with a neutralizing anti-Anx-A1 monoclonal antibody (clone 1B; 20µg/mL) or for 1h with a blocking anti-FPR1 or anti-FPR2 (5µg/ml) or in combination. An irrelevant isotype matched, control antibody (IgG1, ABD Serotec) was also used prior to addition of nedocromil. The chamber was placed under an Eclipse TE3000 microscope (Nikon, Melville, NY, USA) with 40x magnification, and neutrophils (10⁶/ml) were perfused over the endothelial monolayers at a constant rate of 1 dyne/cm² using a syringe pump (Harvard Apparatus Inc., South Natick, MA, USA). After 8 min of perfusion, six random fields were recorded for 10 sec each using a JVC TK-C1360B digital color video camera, ready for off-line analysis. Video sequences were transferred to a computer and loaded into ImagePro-Plus software (Media Cybernetics, Wokingham, Berkshire, UK). Neutrophils were manually tagged and their movements on the endothelium monitored. The total number of
interacting cells was quantified as initial cell capture and further classified as rolling or firmly adherent (cells that remained stationary for the 10 sec observation period).

**FPR-1 and FPR-2 receptors membrane expression in PMNs by FACs analysis**

Aliquots of the PMNs (5x10^4 cells/well) were added to 96-well flat-well plates in duplicates and incubated at 4°C for 1 h in the presence or absence of anti-FPR1 (10µg/ml, R&D, Abingdon, UK), and anti- FPR2 (20µg/ml, Genovac, Germany) in PBS buffer containing 1 mM CaCl_2, 1 mM MgCl_2 and 0.15% BSA; nonspecific binding was minimized by the addition of non-immune mouse immunoglobulin (1.6 mg/ml; Roche, Welwin, UK). The cells were then washed in the buffer and incubated on ice for 30 min with FITC conjugated F(ab)2 fragments of goat anti-rabbit IgG (diluted 1:500). After a further wash in the buffer, cell surface fluorescence was analyzed immediately by flow cytometry. Isotype and unstained controls were also prepared for accurate calibration of the FACS machine. Flow cytometry analysis was assessed using FACS calibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson). FPR1/FPR2 membrane expression was recorded as units of fluorescence, in which the median fluorescence intensity for 10,000 cells was measured in the FL1 green channel (548 nm).

**Static adhesion assay**

HUVEC monolayers were plated in 24 well plates and growth to confluence. After stimulation with TNF-α (10ng/mL) for 4 h, PMNs (10^6 cells) pre-incubated (for 5 min) with either nedocromil, cromoglycate sodium or ketotifen, were added on to the activated monolayer and left to interact for 15 min. Unbound PMNs were removed by washing with warm DPBS. The number of adherent PMNs were then quantified using Alamar Blue (Invitrogen, Paisley, UK) according to the manufacturer’s protocol and the plate was read at
530-560 nm excitation, and 590 nm emission wavelengths. PMN adherence was obtained by subtracting the absorbance acquired from HUVECs alone. The data was normalised and compared to control (untreated cells). The number of adherent cells/field of view was also counted manually by phase contrast microscopy (data not shown) to confirm the results obtained with the colorimetric assay.

Assessment of Ser$^{27}$-Anx-A1-P and PKC activation

PMN extracts were prepared after the detailed incubation with 10nM of the cromones or 2 nM of dexamethasone or 10nM of okadaic acid. To determine if PKC was the sole kinase responsible for Anx-A1 phosphorylation, a specific inhibitor of protein kinase C, PKC$_{19,31}$ peptide (5µM), was incubated with PMNs for 30 min prior to the treatment. The total cellular protein was determined and supernatants analysed by conventional western blotting techniques. Immunodetection was accomplished using different antibodies recognizing either the full-length Anx-A1 protein, (polyclonal anti-Anx-A1 antibody; 1:1000, Invitrogen, Paisley, UK), Anx-A1 phosphorylated on Ser$^{27}$ (polyclonal anti-Ser$^{27}$-Anx-A1 antibody; 1:1000, Neosystem, Strasbourg, France), PKC phosphorylation (polyclonal anti-phospho-PKC antibody; 1:1000, Cell Signalling Technology, Hitchin, UK) and α-tubulin (monoclonal anti-α-tubulin; 1:5000, Sigma-Aldrich, Poole, UK). A horseradish peroxidase-conjugated secondary antibody (1:2000, Sigma-Aldrich, Poole, UK) detected bands related to the proteins of interest and these were revealed by enhanced chemiluminescence.

Assessment of Anx-A1 released onto the PMN cell surface and into supernatants

Anx-A1 protein levels on PMN cell surface and in conditioned medium of PMN incubated with either 10nM of the cromones or 2nM dexamethasone or 10nM of okadaic acid for 5 min at 37°C were determined respectively by flow cytometry and ELISA.
**Flow cytometry:** PMNs were stained with anti-Anx-A1 monoclonal antibody at 10µg/ml (clone 1B) or with its matching isotype control for 1h at 4°C; a final staining with a rabbit anti-mouse IgG (STAR9B; Serotec, Oxford, UK) was conducted. Flow cytometry analysis was assessed using FACS calibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson, San Jose, CA).

**ELISA:** Briefly, 96-well flat-bottomed ELISA plates (Greiner, Gloucestershire, UK) were coated with 1µg anti-Anx-A1 monoclonal antibody 1B in bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing in the bicarbonate buffer, potentially uncoated sites were blocked with 100µL of PBS containing 1% BSA for 1h at room temperature. Sample aliquots (100µL) or Anx-A1 standard solutions (prepared in 0.1% Tween-20 in PBS; concentration range 10- 0.001 µg/mL) were added for 1h at 37°C. After extensive washing in PBS/Tween-20, 100µL of a polyclonal rabbit anti-human Anx-A1 serum (Zymed cat no 71-3400, Invitrogen, Paisley, UK; diluted 1:1000 in PBS/Tween-20) was added for 1h at 37°C before incubation with donkey anti-rabbit IgG conjugated to alkaline phosphatase (1:1000; Sigma-Aldrich, Poole, UK). Colorimetric reaction was obtained by addition of 100µL p-nitrophenyl phosphate (Sigma-Aldrich, Poole, UK; 1mg/mL in bicarbonate buffer, pH 9.6). Absorbance was read at 405nm (with a 620-nm reference filter) in a microplate reader (Titertek™, Vienna, Austria). Anx-A1 levels in the study samples were read against the standard curve and are expressed as ng/ml.

**Analysis of PMNs granules after treatment with cromones by FACs**

Membrane expression of CD11b, CD35, CD66 and CD63 were assessed using anti-CD11b–FITC (20µG/mL), anti-CD35–FITC (25µg/mL), anti-CD66b–FITC (50µg/mL), or anti-CD63–PE (8 µg/mL), respectively (ABD Serotec, Oxford, UK).
**Drugs and other reagents**

Nedocromil sodium was generously supplied by Sanofi-Aventis (Paris, France) and cromoglycate sodium, dexamethasone phosphate and ketotifen were obtained from Sigma-Aldrich (Poole, Dorset, UK). The drugs were made freshly on the day for every experiment. Human recombinant protein Anx-A1 was a kind gift from our colleague Dr F. D’Acquisto.

**Statistical analysis**

*In vitro* analysis were repeated at least 3 times with distinct PMN preparations. For the *in vivo* experimental model, six mice per group were used. Values are expressed as mean ± SEM of *n* observations. Statistical differences between the treated groups were assessed by analysis of variance (ANOVA) followed by Bonferroni’s test for intergroup comparisons. A threshold *p* value ≤ 0.05 was taken as significant.

**Supplemental Figures and Tables**

**Figure I**

To determine if shear stress was required to bring about the inhibitory effects of nedocromil, a static adhesion assay was performed. In addition, we compared the inhibitory effects of the cromones with that of human recombinant (hr) Anx-A1. As expected, hr-Anx-A1 (10nM) strongly inhibited human PMN adhesion onto HUVEC cells (*p*<0.05). A similar result was observed when PMNs were pretreated with the cromones (all tested at 10nM) following a 5 min pre-incubation period. In more detail, cromoglycate produced 45% inhibition (*p*<0.05); nedocromil, 30% inhibition (*p*<0.05) and ketotifen, 20% inhibition (*p*<0.01) on the extent of PMN adhesion to activated HUVECs. Dexamethasone (2nM) was used as a positive control (~30% inhibition; *p*<0.05) in this assay. Interestingly, okadaic acid also inhibited the number
of PMNs adherent to HUVECs, with an effect similar to that attained by the cromones (~40%; p<0.05).

**Figure II**

To identify the intracellular pool of Anx-A1 in PMN that is mobilized during treatment with cromones, different specific antibodies were used to define the granule subsets. Since none of the treatments trigger degranulation of any of the granule subsets (identified as % of positive cells expressing the specific receptor on PMN cell surface), the origin of Anx-A1 is not granular. Therefore, it must be a cytosolic pool that is being activated and this is congruent with our previous work. PMA stimulation of PMN degranulation was used as positive control in this assay.
Figure I: Static adhesion assay

% PMN adhesion inhibition

Vehicle
hr-Anx-A1
Dex
Ket
OA
Ned
Crom
Figure II: Characterization by FACS of PMNs granules after different treatments.

Vesicles, gelatinases

Vesicles

Granules

Azurophils
**Table I.** Hemodynamic parameters of the mesenteric venules recorded in WT mice.

<table>
<thead>
<tr>
<th></th>
<th>Diameter ($\mu$m)</th>
<th>Cell flux (cells/min)</th>
<th>Wall shear rate ($8,000 \times V_{mean}/1.6/Dv$)</th>
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<tbody>
<tr>
<td>Sham</td>
<td>26.3±1.7</td>
<td>8.5±1.5</td>
<td>609.6±33.0</td>
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<tr>
<td>PBS</td>
<td>29.2±0.8</td>
<td>8.4±0.9</td>
<td>306.7±18.7*</td>
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<td>Nedocromil 2mg/kg</td>
<td>27.7±1.6</td>
<td>16.3±3.4*#</td>
<td>515.0±20.2*#</td>
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<tr>
<td>Nedocromil 20mg/kg</td>
<td>25.7±1.4</td>
<td>23.5±5.9*#</td>
<td>486.2±32.6*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (n=4). * P<0.05 relative to sham animals. # P<0.05 relative to PBS animals.
Table II. Hemodynamic parameters of the mesenteric venules recorded in the Anx-A1−/− mice.

<table>
<thead>
<tr>
<th></th>
<th>Diameter (µm)</th>
<th>Cell flux (no. cells/min)</th>
<th>Wall shear rate (8,000xVmean/1.6/Dv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>28.7±1.2</td>
<td>8.0±0.6</td>
<td>383.3±92.7</td>
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<tr>
<td>PBS</td>
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<td>30.5±5.1*</td>
<td>287.5±24.6</td>
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<tr>
<td>Nedocromil 2mg/kg</td>
<td>24.8±0.9</td>
<td>24.5±3.8*</td>
<td>253.0±16.4</td>
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<tr>
<td>Nedocromil 20mg/kg</td>
<td>22.7±1.1</td>
<td>38.5±5.4**</td>
<td>248.5±18.0</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01 relative to sham animals. Data are mean ± SEM (n=4).