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Phosphoinositide 3-kinase γ mediates chemotactic responses of human eosinophils to platelet-activating factor

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ABSTRACT

Background: Eosinophils are characteristic participants in allergic inflammation. The intracellular signalling mechanisms involved in the migration of eosinophils to sites of allergic inflammation are poorly understood. Chemotactic responses of eosinophils to platelet-activating factor (PAF), but not eotaxin, have been demonstrated to be dependent upon the activation of phosphoinositide 3-kinase (PI3K) but the specific isoform of PI3K involved has not been identified.
Objective: To determine the roles of the leukocyte-specific PI3Kγ and PI3Kδ isoforms of PI3K in PAF-induced chemotaxis of human eosinophils.
Methods: Chemotactic responses of the EoL-1 eosinophilic cell line and human peripheral blood eosinophils were measured. The effects of a PI3Kγ-selective inhibitor (5-[2,2-difluorobenzo(1,3)dioxol-5-ylmethylene]-thiazolidine-2,4-dione; AS604850) and gene knock-down of PI3Kγ and PI3Kδ on chemotactic responses were determined.
Results: AS604850 caused a concentration-dependent suppression of chemotactic responses of EoL-1 cells and blood eosinophils to PAF but not eotaxin. Specific siRNAs reduced the expression of PI3Kγ and PI3Kδ in EoL-1 cells. Knock-down of PI3Kγ by siRNA resulted in a 75% inhibition of the chemotactic response to PAF but had no effect on the response to eotaxin. Knock-down of endogenous PI3Kδ by siRNA resulted in a 38% inhibition of the chemotactic response to PAF but had no effect on the response to eotaxin.
Conclusion: PI3Kγ plays a major role in the induction of chemotaxis in PAF-stimulated eosinophils, while PI3Kδ plays a lesser role. Interventions which reduce the activity of PI3Kγ may have therapeutic potential in allergic diseases.

1. Introduction

Eosinophils play an important role in the pathophysiology of allergic diseases. The cellular and molecular mechanisms that specifically regulate the recruitment of eosinophils from the blood to sites of inflammation are complex. In particular, the signalling pathways responsible for evoking migration of human eosinophils and their activation at the inflammatory focus are incompletely understood. Understanding of the mechanisms involved in eosinophil recruitment, activation and survival at sites of inflammation may be useful for the development of novel therapies to control diseases in which eosinophils play a pathophysiological role, such as bronchial asthma, inflammatory bowel diseases, atopic dermatitis and myocarditis [1–4].

Phosphoinositide 3-kinase (PI3K) enzymes provide important signals for cell proliferation, cell survival, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling, superoxide anion radical production, actin reorganisation and chemotaxis [5,6]. PI3K enzymes contribute to the pathogenesis of asthma by influencing the proliferation of airways smooth muscle and the recruitment of eosinophils, and affect the balance between the harmful and protective response in pulmonary inflammation and infection by the modulation of granulocyte recruitment, activation and apoptosis [6,7]. Mammalian class I PI3Ks are a family of homologous kinases that play important roles in multiple cellular processes, including cell motility. A number of studies have shown that PI3Ks are important in the activation of many functions in leukocytes; in particular, the class IA δ and class IB γ isoforms, whose expression is restricted to haematopoietic lineages, are potentially central to the regulation of multiple leukocyte functions [7]. The development of isoform-selective pharmacological inhibitors, targeted gene manipulation and short interfering RNA (siRNA) target

Abbreviations: AS604850, 5-(2,2-difluorobenzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione; ECL, enhanced chemiluminescence; PAF, platelet-activating factor; PI3K, phosphoinositide 3-kinase; siRNA, small interfering RNA.

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validation have facilitated a better understanding of the role that each member of this family of kinases plays in the physiology and pathology of chronic allergic diseases [6,7].

Platelet-activating factor (PAF) and eotaxin (eotaxin-1, CCL11) are important molecules involved in eosinophil recruitment and activation. Both are major chemoattractants for eosinophils, and each acts via a single class of receptor – PAF-R and CCR3, respectively – to induce a variety of responses in the cell [8–10]. Two intracellular signalling pathways have been found to be of particular importance in the recruitment, activation and survival of eosinophils: PI3K and p38 mitogen-activated protein kinase (p38 MAPK) [8,11–13]. The chemotactic response of human peripheral blood eosinophils to PAF has been shown previously to be suppressed by non-selective PI3K inhibitors, wortmannin and LY294002, while the response to eotaxin is unaffected, indicating a differential dependence upon PI3K of responses to different chemoattractants [8]. Similarly, human neutrophil chemoattractants may be divided on the basis of whether their induction of migration is mediated by PI3K or p38 MAPK [14].

While the importance of PI3K in numerous essential physiological processes makes non-selective PI3K inhibition an unlikely therapeutic approach to the treatment of disease, the restriction of expression of the PI3Kδ and PI3Kγ isoforms mainly to leukocytes makes them potentially useful therapeutic targets [7]. A role for PI3K has been demonstrated in animal models of asthma, while gene knock-out experiments have indicated a potential role for PI3Kγ in the cellular and airway changes underlying the features of allergic lung disease [7,15]. We have therefore studied the role of PI3Kγ in eosinophils. Using enzyme-inhibitor and siRNA approaches, we demonstrate a role for PI3Kγ in the PI3K-dependent, PAF-induced chemotaxis of human eosinophils.

2. Materials and methods

2.1. Cells

The human eosinophilic leukaemia cell line, EoL-1, was purchased from the European Collection of Cell Cultures (Porton Down, UK). Cells were differentiated to an eosinophilic phenotype as described previously [16]. Human peripheral blood eosinophils were isolated from non-asthmatic volunteers as described previously [17]. Blood donors gave informed consent to the use of their cells, and the project was approved by the North Staffordshire Local Research Ethics Committee (reference 06/Q2604/15).

2.2. Gel electrophoresis and Western blotting

Proteins were extracted from EoL-1 or peripheral blood eosinophil pellets in radioimmunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF), sodium vanadate and protease inhibitors (Santa Cruz Biotechnology, Heidelberg, Germany). Total protein was assayed by BCA, and 30 μL portions of lysate containing 40 μg (EoL-1 cells) or 27 μg protein (peripheral blood eosinophils) were used in experiments. Proteins were separated by electrophoresis (180 V for 45 min) on SDS-polyacrylamide (12%) gels (Bio-Rad, Hemel Hempstead, UK) and blotted onto HyBond enhanced chemiluminescence (ECL) membranes (Amersham Biosciences, Little Chalfont, UK) at 30 V for 24 h. PI3Kγ and PI3Kδ were detected using a rabbit polyclonal anti-PI3K p110γ IgG (H-199; Santa Cruz Biotechnology) or anti-PI3K p110δ IgG (ab1678; Abcam, Cambridge, UK) at a dilution of 1:200 or 1:3,333, respectively, followed by horseradish peroxidase-labelled goat anti-rabbit IgG (Sigma-Aldrich, Poole, UK) and ECL (Amersham Biosciences).

2.3. Small interfering RNA (siRNA)

Experiments with siRNA were conducted only with EoL-1 cells: blood eosinophils did not survive for long enough in culture to allow effective transfection. Three different PI3Kγ gene-specific predesigned siRNAs (ID 143807 for exon 2, 143809 for exon 5 and 12064 for exon 11), one PI3Kδ siRNA (ID 143975 for exon 5) and negative control siRNA were purchased from Ambion/Applied Biosystems (Warrington, UK). All siRNAs were HPLC purified, annealed and ready for use. Transfection efficiency of siRNA was determined using Cy3-labelled siRNA duplexes. The transfection efficiency in the experiments reported here was 80–90%.

On the day of transfection, 10^6 differentiated EoL-1 cells were centrifuged and washed twice in Opti-MEM I reduced-serum medium (Invitrogen Ltd., Paisley, UK) prior to resuspension in 400 μL Opti-MEM I. A series of experiments was conducted to determine optimal transfection conditions (see Results, below), beginning from the conditions for DNA transfection published by Ohyama et al [18]. On the basis of the results of these, cells for functional experiments were incubated with 20 nM siRNA at 37 °C for 30 min prior to poration in a 0.4 cm electroporation gap cuvette. Cells were electroporated at 274 V and 1,150 μF using a Bio-Rad gene pulser. Cells were allowed to stand at 37 °C for 30 min prior to transfer to 6-well plates containing Iscove's modified Dulbecco's medium (Sigma-Aldrich, Poole, UK) supplemented with 2 mM L-glutamine and 20% heat-inactivated foetal calf serum. Transfected cells were used for experiments after 72 h.

2.4. Chemotaxis assay

PAF- and eotaxin-induced chemotaxis of untreated, inhibitor-pretreated, sham-transfected or specific siRNA-transfected cells was measured in a fluorescence-based 96-well blind-chamber chemotaxis assay, as described previously [8]. Non-transfected cells were pre-incubated for 30 min at 37 °C with or without 1 pM–10 μM 5-[2,2-difluorobenzo(1,3)dioxol-5-ylmethylene]-thiazolidine-2,4-dione (AS604850, also known as PI3Kγ inhibitor II; Merck Biosciences, Nottingham, UK). Transfected cells were equilibrated without AS604850 under the same conditions. Cells in control or AS604850-containing medium were then loaded into the upper chambers of the apparatus at 2×10^5/well and incubated for 120 min (EoL-1 cells) or 60 min (blood eosinophils) prior to quantification of migrated cells.

2.5. Statistical analysis

Data are expressed as mean and standard error of the mean (SEM) from the indicated numbers of experiments. All statistical analysis was performed using InStat 3.10 (GraphPad Software, San Diego CA, USA). As cell migration responses were log-normally distributed, data were log-transformed prior to statistical analysis. Comparisons between siRNA-transfected and sham-transfected cells were performed using the paired *t* test. For comparisons of multiple groups (e.g. varying concentrations of enzyme inhibitor), repeated-measures analysis of variance was followed by *post hoc* pairwise comparisons with control (no inhibitor) cells using Dunnett's test for multiple comparisons. A probability (P) <0.05 was defined as significant throughout.

3. Results

3.1. Expression of PI3Kγ and PI3Kδ isoenzymes

The expression of the p110 γ and δ catalytic units of PI3K was confirmed in EoL-1 cells and human peripheral blood eosinophils (Figs. 1A, 2A, 3, 4, 5A).

3.2. Effects of AS604850 (PI3Kγ inhibitor II) on eosinophil chemotactic responses

PAF and eotaxin both induced migration of EoL-1 cells and peripheral blood eosinophils. In initial concentration–response experiments, optimal concentrations were 100 nM PAF (net migration 21,600 ± 5390 cells/