



Preliminary report

Tyrosine kinase inhibition is an important factor for gene expression of CRTH2 in human eosinophils and lymphocytes: A novel mechanism for explaining eosinophils recruitment by the neuro-immune axis in allergic rhinitis



AE El-Shazly ^{a,b,*}, P Roncarati ^b, M Lejeune ^b, PP Lefebvre ^a, P Delvenne ^b

^a Department of Otolaryngology and Head and Neck Surgery, Liege University Hospital-CHU, Liege, Belgium

^b Department of Pathology, Laboratory of Experimental Pathology, University of Liege, Liege, Belgium

ARTICLE INFO

Article history:

Received 9 January 2017

Received in revised form 12 February 2017

Accepted 14 February 2017

Available online xxxx

Keywords:

Allergic rhinitis

CRTH2

Late phase reaction

Tyrosine kinase inhibitors

Leukocytes

ABSTRACT

We recently shown a novel neuro-immune competition between vasoactive intestinal peptide (VIP) and PGD2 for CRTH2 receptor, and that genistein augmented VIP and PGD2-induced eosinophil chemotaxis. However, there are neither studies on the CRTH2 gene expression in allergic rhinitis (AR) nor in the effect of tyrosine kinase inhibitors in CRTH2 gene regulation.

Our Objectives were to study the gene expression modulation of CRTH2 receptor in AR patients and the effect of tyrosine kinase inhibitors (TKIs) on CRTH2 gene modulation. Nasal provocation tests, ELISA, qRT-PCR, western blot, flow cytometry and chemotaxis assays in modified micro-Boyden chambers, were all used, to achieve our objectives. Herein we show that AR patients increased the amounts of VIP and PGD2 in their nasal secretions in the early phase reaction, however CRTH2 gene expression from leukocytes recovered in their nasal secretions was upregulated only during the late phase reaction. The TKIs; Genistein, Erbstatin and Herbimycin A, induced the gene expression of CRTH2 and increased the protein content of CRTH2 in both human lymphocytes and eosinophils. This was functional as PGD2/VIP-induced eosinophil chemotaxis was augmented by the TKIs and inhibited by pervanadate, the tyrosine phosphatase inhibitor. These results open channels for therapeutic modalities targeting CRTH2 molecules in AR.

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1. Introduction

In allergic rhinitis inflammation, lymphocytes and eosinophils play pivotal role, being the chief inflammatory cell types infiltrating the nasal mucosa. Recruitment of eosinophils in large numbers into the allergic inflamed nose during the late phase reaction entails a complex process of directed cell migration against concentration gradient of the chemokine (chemotaxis). For this to happen, the chemokine interaction with its specific receptor mediates signal transduction via Ca^{2+} mobilization, protein kinase C, and heterotrimeric GTP-binding proteins [1–3] or Kinases and phosphatases, adaptor proteins, and a small GTP-binding proteins [4–5].

CRTH2 is expressed on eosinophils, basophils and T Helper Type 2 lymphocytes. CRTH2 gained a lot of attention as promotor for PGD2-induced eosinophilia in allergic airway diseases [6–10]. In an allergic

rhinitis murine model, it has been demonstrated that the PGD2-CRTH2 interaction is elevated following pollen sensitization. Additionally, CRTH2 mRNA in nasal mucosa was significantly elevated in Cry j 1-sensitized mice [11]. Moreover, in nasal tissue, ligation of PGD2 to CRTH2 appeared to be selectively involved in eosinophil recruitment [12]. Recently we showed that VIP amounts are significantly higher in AR patients nasal secretions compared to control subjects and that VIP competes with PGD2 for CRTH2 receptor signaling for eosinophil chemotaxis [13]. This highlighted a novel neuro-immuno-axis in recruiting eosinophils via CRTH2.

Tyrosine kinases is an important factor for cell differentiation and mediates many cellular biological effects. Interestingly, our previous biochemical assays showed that tyrosine kinase activity in human eosinophils is an essential down-stream signal that promotes eotaxin induced eosinophil chemotaxis via CCR3 receptor [14]. On the other hand, tyrosine kinase inhibition was not involved in PGD2/VIP-induced eosinophil chemotaxis, but rather augmented eosinophil chemotaxis against VIP and PGD2 [13,15,16].

However, the role of tyrosine kinases in PGD2/VIP-CRTH2 interaction is not fully understood. Further no study has yet addressed the

* Corresponding author at: Department of Otolaryngology and Head and Neck Surgery, Rhinology Division, Liege University Hospital, Sart Tilman, 4000 Liege, Belgium.

E-mail address: amrel_shazly@hotmail.com (A.E. El-Shazly).

¹ Experimental Rhinology unit, Laboratory of Experimental Pathology.

functional gene expression of CRTH2 in allergic rhinitis patients and the role of tyrosine kinase inhibitors in augmenting eosinophil chemotaxis via CRTH2 stimulation.

2. Materials and methods

2.1. Patients and nasal allergen challenge

2.1.1. Inclusion criteria

- AR patients' mono-sensitized to HDM allergen proved by allergy skin test and radioallergosorbent test (RAST).
- AR patients' mono-sensitized to HDM and were not taking any anti-allergy pharmacotherapy at least 2 months before the study.
- AR patients' mono-sensitized to HDM allergen and presented with mild to moderate persistent AR (nasal obstruction, sneezing and rhinorrhea that does not affect sleep pattern, daily activities or absence from work).

2.1.2. Exclusion criteria

- Pregnant women.
- Children.
- AR patients sensitized to other allergens than HDM.
- Patients with severe AR symptoms that affect sleep pattern, daily activities or absence from work.
- AR patients who were taking already nasal corticosteroids, antihistamine or anti-leukotrienes or in immunotherapy program.
- AR patients with associated asthma.
- AR patients with acute rhinosinusitis.
- AR patients taking B-blockers

2.1.3. Patients' nasal provocation

Thirty patients were recruited to this study after obtaining their consent and the approval of our university hospital ethical committee (2016/137). Nasal provocation tests were done as a part of monitoring their disease activity or prior to immunotherapy program. Their nasal secretions were collected before challenge and either during 30 min or 8 h after challenge. The collected nasal secretions were first weighted and diluted three times with DPBS (Lonza BioWhittaker, Belgium). After centrifugation at 872g for 8 min at 4 °C, the cell pellet was subjected to total RNA extraction as described below, while the supernatants, were collected and stored at –80 °C until they were tested by ELISA according to the manufacturer protocol for the contents of VIP (Phoenix Europe-Germany) and PGD2 (Bertin Pharma, France).

2.2. Tyrosine kinase inhibitors (TKIs)

Briefly, human eosinophils from peripheral blood buffy coats were isolated using hypotonic lysis and centrifugation. A purity of >95% was obtained. Lymphoprep™ was used for the preparation of pure lymphocyte suspensions from human blood.

On the day of the experiment, cells were incubated with either different doses of Genistein (Sigma-Aldrich, Germany); Herbimycin A (Enzo Life Sciences, United States) and Erbstatin (Santa Cruz Biotechnology, United States), for periods of 1 h, 6 h and 24 h at 37 °C. Cells were collected by centrifugation at 1200g for 5 min and were lysed by adding Lysis Solution (supplied with the NucleoSpin RNA kit).

2.3. Quantitative RT-PCR

The total RNA of the cells was extracted using a NucleoSpin RNA kit (Macherey-Nagel, Duren, Germany). cDNA of the samples was prepared from the same starting amount of RNA using a SuperScript First-Strand Synthesis System (Invitrogen). qPCR was conducted using Master Mix

and Probe in a ABI 7900HT Sequence Detection System (Applied Biosystem). GAPDH was used as control gene. The changes of mRNA level compared with untreated sample were calculated according to the manufacturer's instructions. Primers and probes from The Universal ProbeLibrary Set, (Roche, Mannheim, Germany). CRTH2 with probe 43 generating a 95-bp fragment and GAPDH with probe 60 generating a 66-bp fragment, as follow:

Gene	Primer	Probe n°
CRTH2	5'-CCTGTGCTCCCTCTGTGC-3'	43
	5'-TCTGGAGACGGCTCATCTG-3'	
GAPDH	5'-AGCCACATCGCTCAGACAC-3'	60
	5'-GCCAATACGACCAATCC-3'	

2.4. Western blotting

For preparation of whole cell extracts, cells were pelleted by centrifugation and washed twice with PBS. The cell pellets were resuspended in cold RIPA lysis buffer supplemented with protease inhibitors (Complete, Roche Applied Science) and subsequently swirled for 30 min on ice. The extract was then centrifuged at 14,000 × g for 15 min at 4 °C. The supernatants were analyzed for protein concentration by the Micro BCA Protein Assay kit (Thermo Scientific).

The antibodies used for Western blot were rabbit anti-CRTH2 antibody (1/1000, PA5-20332, Life Technologies), rabbit anti-Actin antibody (1/5000, A2066, Sigma), donkey anti-rabbit HRP (1/2000, NA934V, GE Healthcare) and antiphosphotyrosine antibody (abcam ab 10321).

All reagents and equipment used for electrophoresis, and transfer of protein were used according to the manufacturers' instructions regarding the NuPage system (Invitrogen).

Protein bands were visualized with enhanced chemiluminescence with the ECL system (Pierce). Blots were quantified using Quantity One software (Biorad).

2.5. Chemotaxis assay

Chemotaxis assays were performed in triplicate in a 48-well microchemotaxis Boyden chamber incubated in 5% CO₂ at 37 °C for 90 min. Aliquots of 29 µL of VIP (Phoenix Pharmaceuticals), or PGD2 (Cayman chemical) chemotactic agent were placed in the lower wells, and 50 µL of an eosinophil cell suspension (10⁶ cells/mL) was placed in the upper wells. The two chambers were separated by a 5 µm pore polycarbonate membrane (Nuclepore, Whatman, Middlesex, UK). Migrated cells adhering to the lower surface were counted in 5-selected high power fields/well under a light microscope (5hpf; ×400). As for blocking experiments, the eosinophils were pre-treated with pervanadate (100 mM sodium orthovanadate with 100 mM hydrogen peroxide), for 30 min at 37 °C. For TKIs experiments, the eosinophils were pretreated with TKIs for 60 min at 37 °C. Cells were then washed twice, before their viability was rechecked by Trypan blue staining, which was always >98%. Cells were re-suspended in buffer medium and their chemotaxis was checked as stated above.

2.6. Flow cytometry analysis (FACS)

CRTH2 surface expression on eosinophils was analyzed by FACS (FACS CANTO II BD Systems). Briefly, stimulated (TKI) or not stimulated (buffer) cells were fixed with 4% paraformaldehyde for 15 min. The cells were then washed and incubated with the CRTH2 antibody (BD-Pharmingen), for 60 min in the dark, on ice. After two additional washes, cells (10⁵ cells/FACS plot) were conserved in paraformaldehyde 1% and then analyzed for their fluorescence intensity.

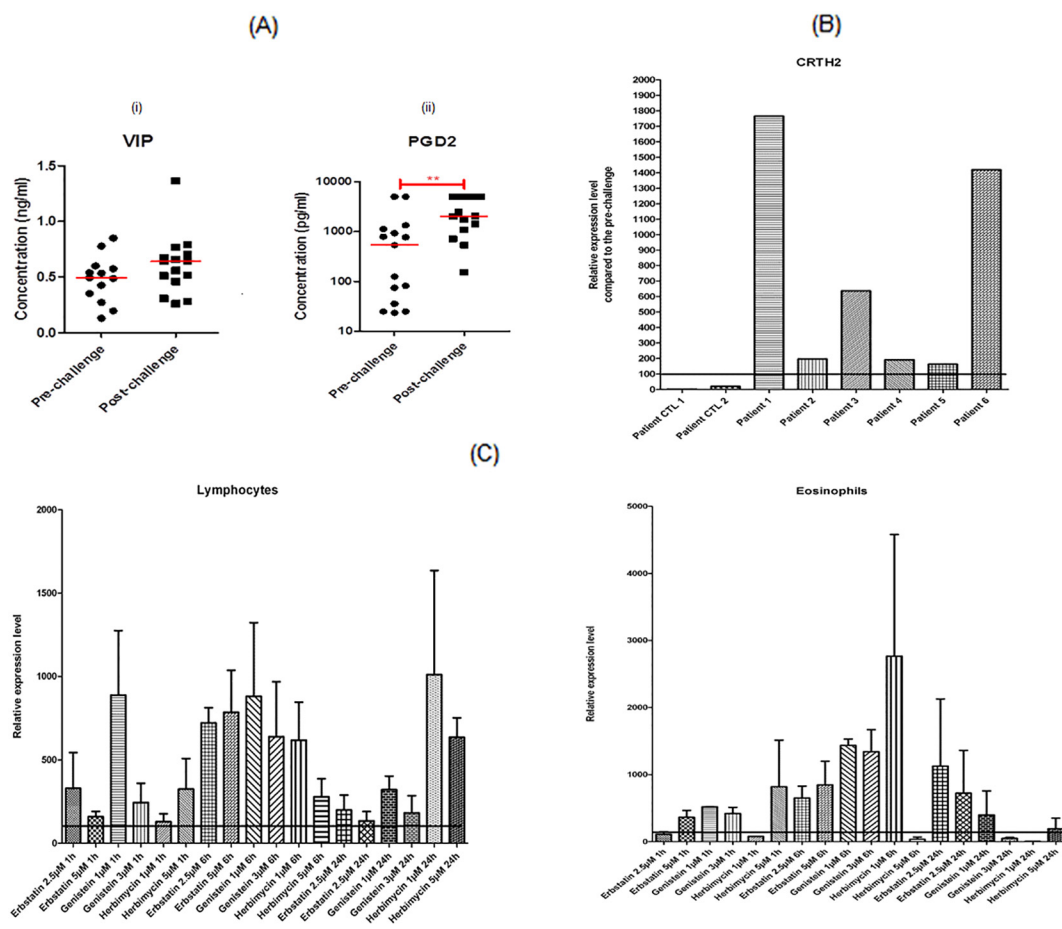


Fig. 1. (A) Amounts of VIP (i) and PGD2 (ii) recovered from nasal secretions of 14 AR patients' monosensitized to HDM allergen (mean age 29.7 years old; 13M:1F) before and 30 min after nasal provocation with diluents of HDM allergen (Alyostal-Stallergenes-41018-1). Nasal provocation was performed precisely as described before using spray technique according to the manufacturer instruction. Asterisks indicate statistical significance by Wilcoxon test. (B) Relative quantification of mRNA CRTH2 levels (normalized to GAPDH) recovered from nasal secretions of 8 allergic patients monosensitized to HDM allergen (mean age 28.5 years; 6M:2F) who were challenged with HDM allergen diluents; 2 patients acted as control and they were challenged with the diluent only. Horizontal line as 100% represented the level expression for the pre-challenge. (C) Percentage of CRTH2 level in peripheral blood lymphocytes and eosinophils after TKIs treatments compared to the lymphocyte and eosinophil untreated represented as 100% at the respective time-points. The mRNA levels of lymphocytes and eosinophils were measured using qRT-PCR. N = 6.

3. Results

3.1. VIP and PGD2 amounts increases in nasal secretions of mono-sensitized AR patients to HDM during the early phase reaction while CRTH2 gene expression by human leukocytes recovered in nasal secretions is up-regulated in the late phase reaction

VIP and CysLTs are significantly elevated in nasal lavage fluid from symptomatic AR patients compared to healthy controls [13,17–19]. We first measured the amounts of VIP and PGD2 recovered in nasal secretions from AR patient's mono-sensitized to HDM allergen during the early phase reaction, before and after nasal challenge. As can be seen in Fig. 1A(i and ii), challenging the nasal mucosa of AR patients with diluents of the allergen extract, resulted in an increase in VIP and significant increase in PGD2 amounts in nasal secretions, from the baseline (before challenge). These results indicate that these mediators which are already elevated in allergic rhinitis patients are capable to be further up-regulated after nasal challenge with the allergen, indicating a potential role(s) for the parasympathetic mediator VIP and the eicosanoid mediator PGD2, in the pathophysiology of allergic rhinitis to HDM allergen. As VIP competes for chemotaxis signaling with PGD2 via the CRTH2 receptor on human eosinophils [13], we next measured the CRTH2 receptor gene expression in the leukocytes recovered in nasal secretion during the early phase reaction.

There was no significant increase of CRTH2 gene expression after nasal challenge in 8 AR patients' mono-sensitized to HDM during the early phase (data not shown). However, the gene expression was clearly increased during the late phase reaction in another 6 AR patients also mono-sensitized to HDM (Fig. 1b). This result indicates that the up-regulation of CRTH2 in leukocytes takes place during the late phase reaction. As we reported earlier that the tyrosine kinase inhibitor genistein primed human eosinophils and eosinophil cell line (Eol-1) to exaggerated chemotaxis response against VIP and PGD2 [13,15,16], we next studied the effect of different tyrosine kinase inhibitors on the CRTH2 expression by peripheral blood human leukocytes; lymphocytes and eosinophils.

3.2. Augmentation of CRTH2 gene expression of human leukocytes by erbstatin, herbimycin A and genistein measured by RT-PCR

In our preliminary experiments we tested wide range of doses for each inhibitor and the optimal doses were chosen as shown in Fig. 1c. At the optimal time course of 6 h all three tested inhibitors were able to induce the up-regulated expression of CRTH2 in lymphocytes and eosinophils. This activity started as early as 1 h and lasted up to 24 h. In lymphocytes, genistein at the dose of 1 μ M was the most powerful inducer of CRTH2 gene expression at 1 h while herbimycin A was the most powerful inducer of CRTH2 at concentration of 1 μ M at 24 h. In

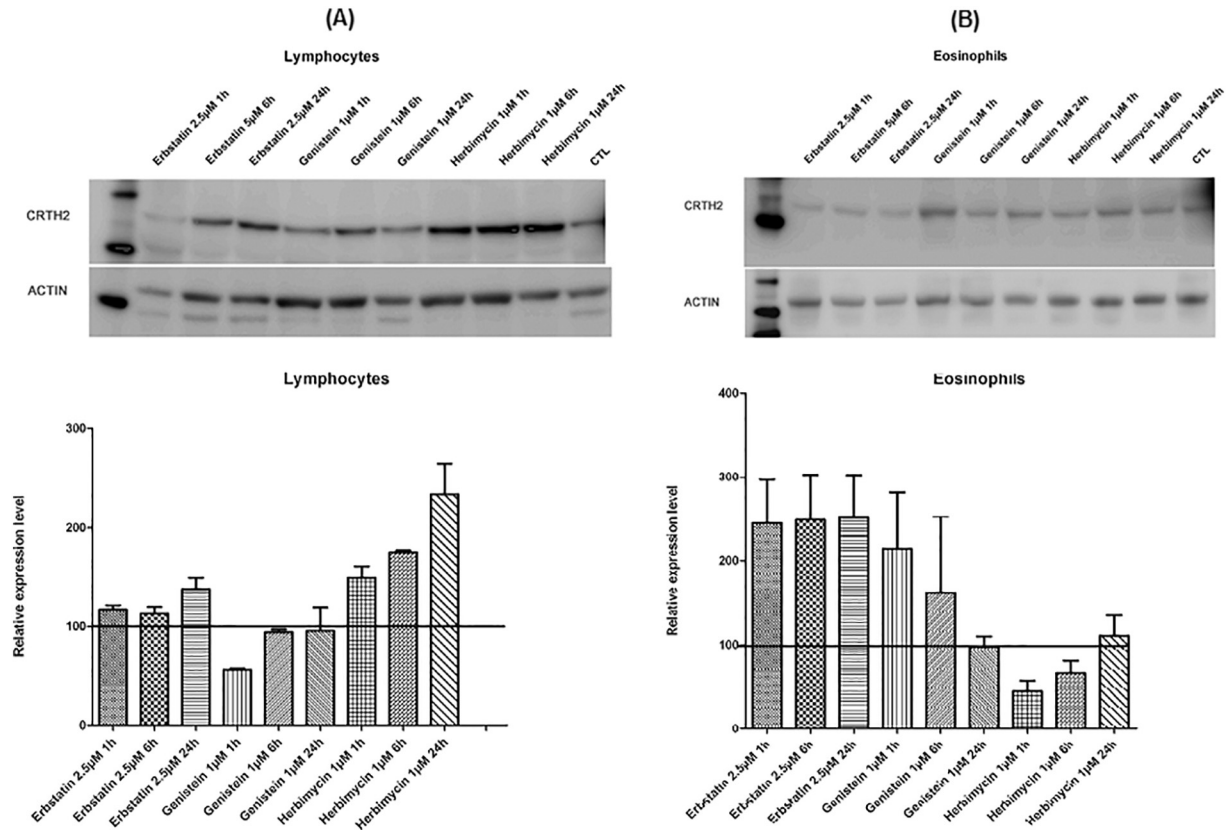


Fig. 2. (A) Effects of different TKIs on the expression level of CRTH2 in lymphocytes. Western Blots were performed on whole cell extracts. Lower panel shows graphs representing the quantification of western blots experiments, the lymphocytes without treatment represented as 100%. (B) Effects of different TKIs on the expression level of CRTH2 in eosinophils. Western Blots were performed on whole cell extracts. Lower panel shows graphs representing the quantification of western blots experiments. Eosinophils without treatment are represented as 100%.

eosinophils, herbimycin A was the most powerful inducer of CRTH2 gene expression at the dose of 5 μM at 1 h while erbatatin at 2.5 μM concentration was the most powerful inducer of CRTH2 gene expression at 24 h. These results indicate the importance of tyrosine kinase down-regulation for the gene up-regulation of CRTH2 receptor.

3.3. Augmentation of CRTH2 receptor protein expression of human leukocytes by erbatatin, herbimycin A and genistein

Next we performed western blot experiments to see whether the optimal doses of TKIs that induced CRTH2 gene expression were also

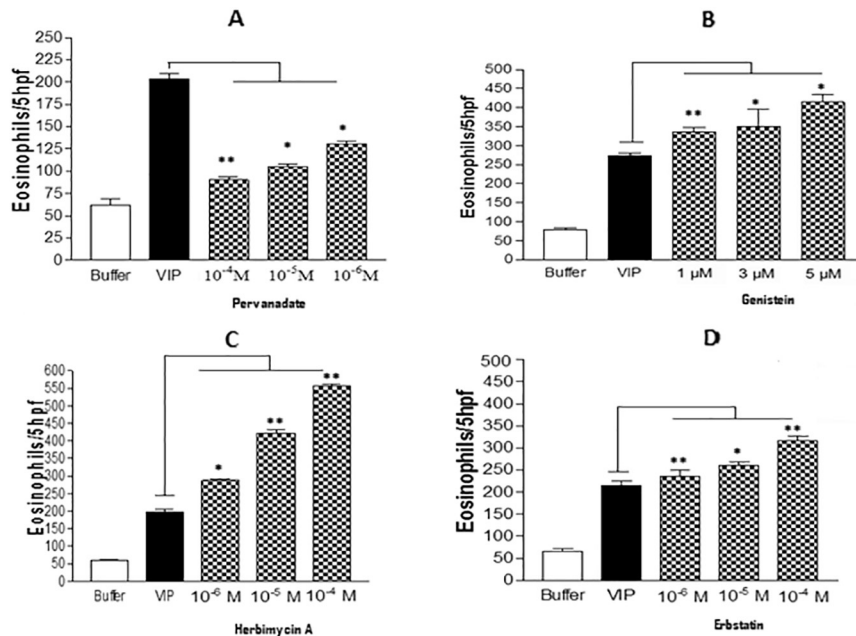


Fig. 3. Chemotactic activity of 10^{-7} M VIP on human eosinophils in presence of (A) Pervanadate (B) Genistein (C) Herbimycin A and (D) Erbatatin. Asterisk indicates $^*P < 0.05$ and $^{**}P < 0.001$, by paired *t*-test. Results are means $\pm$ SEM of 8 independent experiments performed in triplicate.

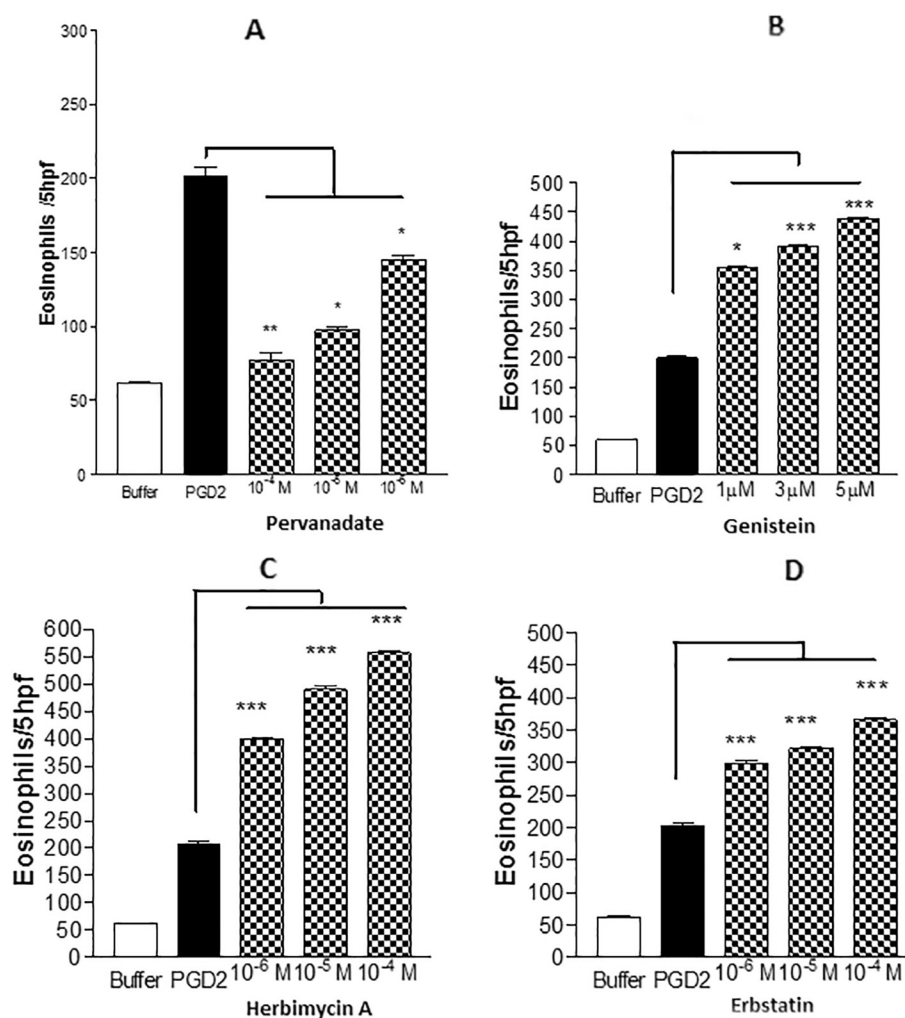


Fig. 4. Chemotactic activity of 10^{-9} M PGD2 on human eosinophils in presence of (A) Pervanadate (B) Genistein (C) Herbimycin A and (D) Erbstatin. Asterisk indicates $*P < 0.05$ and $***P < 0.0001$, by paired *t*-test. Results are means $\pm$ SEM of 8 independent experiments performed in triplicate.

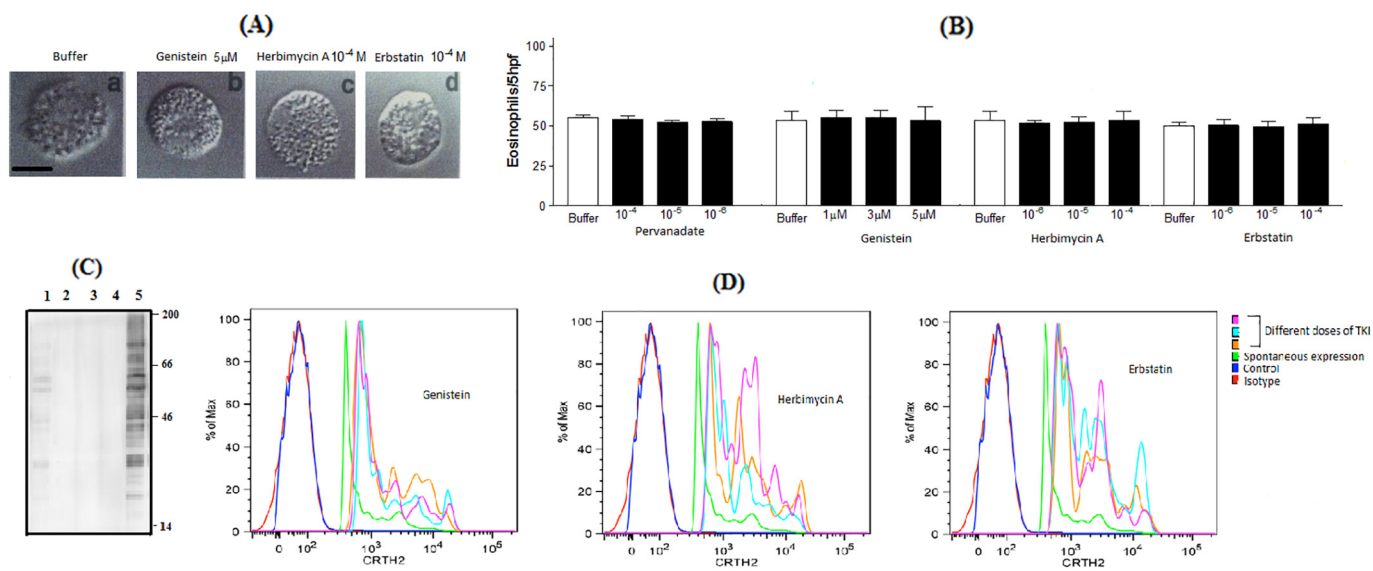


Fig. 5. Relationship between the effect of TKIs and pervanadate in tyrosine phosphorylation and morphological changes. (A) Cells were fixed and imaged by Normarski differential-interference-contrast. Eosinophils were incubated with buffer only (a) or various TKIs (c–d) for 1 h in absence of VIP or PGD2. Bar, 5 μm. Images are of one experiment representative of 3, all showing similar results. (B) Random migration of human eosinophils treated by different doses of TKIs and pervanadate (N = 6). (C) Equal amounts of whole cell lysates were analyzed by western blotting with the 4G10 mAb for phosphotyrosine. Molecular size markers are indicated on the right (kDa). Stimulation was as follows: Lane 1 = Buffer; 2 = Erbstatin 2.5 μM, 3 = Genistein 1 μM, 4 = Herbimycin A 1 μM and 5 = Pervanadate 250 μM. (D) Flow cytometry. CRTH2 surface expression in human eosinophils by different doses of TKIs (used in the chemotaxis assay). Histograms are of one experiment representative of five showing similar results.

able to increase the protein synthesis on human leukocytes. As seen in Fig. 2, the different TKIs also increased the amount of CRTH2 protein in both lymphocytes and eosinophils. The maximum protein content was achieved by herbimycin A in lymphocytes and by erbstatin in eosinophils.

3.4. Functional surface expression of CRTH2 by human eosinophils

Finally, to explore whether these augmentations of CRTH2 gene and protein expression by TKIs were functional, chemotaxis assays were performed to check the eosinophilotactic activity of VIP and PGD2 in the presence and absence of genistein, erbstatin and herbimycin A. As seen in Figs. 3 & 4(B–D) all three TKIs augmented significantly the VIP/PGD2-induced eosinophil chemotaxis. Interestingly, pervanadate the tyrosine phosphatase inhibitor significantly inhibited PGD2/VIP-induced eosinophil chemotaxis (Figs. 3A & 4A). To exclude the possibility of activation of eosinophils random migration and cytoskeletal changes by TKIs and pervanadate, chemotaxis experiments and confocal images were performed. As seen in Fig. 5A & B, eosinophils pretreatment with different TKIs neither enhance the basal migratory capacity of the cells in the absence of VIP & PGD2, nor induced cell shape changes. Nonetheless, tested TKIs and pervanadate were effectively able to inhibit and augment several protein tyrosine kinase residues phosphorylation in human eosinophils respectively, as seen in Fig. 5C. However, flow cytometry demonstrated upregulation of CRTH2 surface expression in eosinophils in response to TKIs as seen in Fig. 5D.

Taken collectively, these results may indicate that CRTH2 surface expression is regulated by the delicate balance between tyrosine kinase and phosphatase activities (Fig. 5c) in an independent fashion to cytoskeletal changes (Fig. 5A & B) resulting in a novel priming effect with exaggerated eosinophil migratory response against VIP and PGD2 (Figs. 3 & 4).

4. Discussion

In the current study we provide the first evidence that CRTH2 gene expression in human lymphocytes and eosinophils takes place in the late phase reaction of allergic rhinitis patients and this requires at least in part the inhibition of tyrosine kinase activity. The fact that nasal leukocytes' CRTH2 was upregulated only in the late phase reaction, 8 h after the allergen challenge, identifies the important role of CRTH2 in recruiting eosinophils in large number to the inflamed allergic nose. This is a hallmark in the late phase reaction of airway allergy.

After the allergen challenge and the induction of the allergic cascade, there are several mediators that are released in the allergic nasal tissue including VIP and PGD2; the only described ligands to date for CRTH2. This pool of mediators is key player in the promotion of allergic inflammation. It is possible that some of these mediators modulate tyrosine kinase/phosphatase activity in lymphocytes and eosinophils, and thus regulating CRTH2 expression in these cells.

Our results indicate clearly that tyrosine kinase inhibitors at least in part are capable of inducing CRTH2 gene expression and CRTH2 protein synthesis. This was functional as our chemotaxis assays demonstrated exaggerated chemotactic response of human eosinophils against PGD2 & VIP by genistein, herbimycin A and erbstatin treatments and significant inhibition by pervanadate, the tyrosine phosphatase inhibitor. This is probably through upregulation of CRTH2 surface expression as demonstrated by flow cytometry in Fig. 5C. Of note that none of the TKIs tested neither changed eosinophils shape nor increased their random migration, indicating a specific effect of tyrosine kinase activity in CRTH2 dynamics in these cells that is independent to cytoskeletal changes and random migration.

The ability of TKIs in inducing CRTH2 expression could be related to the c-terminal of CRTH2 receptor that is important molecular determinant in the down-stream signaling cascade activation [20]. This requires further studies to prove it or disprove it. Additionally, further studies are

needed to explore which specific tyrosine kinase(s) is involved in the CRTH2 gene expression that is beyond the scope of the current communication.

In conclusion, we propose that tyrosine kinase inhibition is a novel mediator of inflammation. In one hand, as we reported earlier, it can attenuate eosinophilic inflammation mediated via CCR3 [14] and on the other hand, as reported herein, it may at least in part be an important factor in inducing CRTH2 dynamics and functionality, and thus potentiating eosinophilic inflammation. If so, this may provide a novel mechanism for the pathophysiology of the neuro-immuno-axis in allergic rhinitis, and open channels for therapeutic modalities aiming at treating inflammatory cells in allergic rhinitis.

Competing interests

None of the authors has any potential financial conflict of interest related to this manuscript.

Acknowledgments

This study was supported by fund from CHU de Liege-FIRS, 2014/15.

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