



Pulmonary, gastrointestinal and urogenital pharmacology

Cytokine production by PBMC and serum from allergic and non-allergic subjects following in vitro histamine stimulation to test fexofenadine and osthole anti-allergic properties

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ABSTRACT

FXF is a third-generation antihistamine drug and osthole is assumed a natural antihistamine alternative. This paper compares peripheral blood mononuclear cell (PBMC) incubation with FXF and osthole, by studying FXF, osthole and histamine cytokine secretion in PBMC in vitro cultures. Mabtech kits determined the interleukins IL-1 β , IL-4, IL-10, IL-13 and TNF- α . The influence of the above active substances on cytokine secretion in PBMCs and serum was assessed: cytokines were IL-1 β , IL-4, IL-10, IL-13 and TNF- α ; and cytokine levels secreted by untreated PBMCs in pure culture medium formed the absolute control (ctrl).

We determined that osthole affects PBMC cytokine secretion to almost precisely the same extent as FXF (IL-1 β , IL-4, IL-10 and TNF). In addition osthole had greater IL-13 blocking ability than FXF. Moreover, we observed significantly decreased IL-4 level in histamine/osthole treatment compared to histamine alone. Meanwhile, FXF not significantly decrease the level of IL-4 increased by histamine. This data indicates osthole's strong role in allergic inflammation.

All results confirm our hypothesis that osthole is a natural histamine antagonist and therefore can be beneficially used in antihistamine treatment of conditions such as allergies.

1. Introduction

The link between allergic diseases and abnormal patterns of immune development has stimulated efforts to define the precise patterns of cytokine dysregulation associated with specific atopic phenotypes (Sismanopoulos et al., 2012). The immune system has developed efficient peripheral tolerance mechanisms to avoid chronic cell activation and inflammation against nonpathogenic antigens from ingestion, inhalation and through the skin (Yan and Hansson, 2007).

Allergic diseases are caused by an aberrant immune response mediated through a key effect for the Th2 cell and an associated cytokine pattern including interleukins (ILs) -4, -5 and -13 (Gabay et al., 1997; Hobbs et al., 1998; Mosmann, 1996; Ying et al., 1991). Clinical manifestations of mast cell- and basophil-dependent allergic reactions include not only rapidly evolving, immediate type reactions, but also more protracted "late-cutaneous" reactions which are mast cell dependent and associated with an influx of neutrophils, macrophages and activated lymphocytes and eosinophils (Bochner et al., 1995;

Hoekstra et al., 1997; Prescott et al., 1998).

Rapidly released mast cell mediators and numerous cytokines produced by these cells are reported to induce and sustain this response. Cytokines produced by human mast cells include molecules initiating and boosting the TH-2 type immunological reactions, and also a broad spectrum of pro-inflammatory, growth-promoting and chemotactic responses for diverse cell types. These mediators include ILs-1, 3, 4, 5, 6, 10, 13 and 16, tumor necrosis factor (TNF)- α , granulocyte macrophage colony stimulating factor (GM-CSF), transforming growth factor (TGF)- β , platelet derived growth factor (PDGF), special isoforms of vascular endothelial growth factor (VEGF), nerve growth factor (NGF), chemokines IL-8 and I 309, and macrophage chemotactic protein (MCP)-1: "regulated-upon-activation normal T cell expressed and secreted" (RANTES), macrophage inhibitory proteins (MIP)-1a and b and lymphotactin (Borish et al., 1996; Bradding et al., 1994; Gabay et al., 1997; Lummus et al., 1998; Zipperlen et al., 2005; Metcalfe et al., 1997). *In vitro* and animal studies suggest a cytokine role in initiation of allergic inflammation (Olsen et al., 2004).

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Osthole is a natural coumarin, first derived from the *Cnidium* plant, *Fructus Cnidii*, and it is reported to strengthen the immune system, enhance male sexual function and relieve rheumatic pain. (Hoult and Pava, 1996; Wang et al., 2007). Modern research suggests that osthole also has antioxidant, anticancer, anti-inflammatory and immunomodulatory properties (Fiorentino et al., 1991; Hampe et al., 1999; Pyo et al., 2003; Simpson and Jarvis, 2000). Hence, we presume that osthole inhibits secretion of pro-inflammatory IL after allergic inflammation.

To test this hypothesis, we measured cytokine IL-1 β , IL-4, IL-10, IL-13 and TNF- α secretion in medium and in the serum of allergic and non allergic patients after PBMC stimulation by histamine to determine fexofenadine (FXF) and osthole anti-allergic properties. We also compared the levels of measured IL between histamine and histamine/FXF and histamine/osthole to investigate FXF and osthole anti-histamine effects. A very important by-product of these results; was to discover if osthole has the same effect as fexofenadine in experimental conditions; and hence, can osthole equal beneficial effect of FXF and replace it in clinical practice?

2. Material and methods

2.1. Study participants

Peripheral blood mononuclear cells (PBMC's) were collected from 30 healthy people (age: $M=30.90$ years, $SD=4.95$ years) (control group) and 30 people diagnosed with allergy (age: $M=31.10$ years, $SD=4.94$ years). People with fever, infections and skin problems, those taking steroids or antibiotics and current or previous smokers were excluded from the study. The patients were allowed β_2 antagonists when necessary, but no medications were taken 24 h prior to blood collection and all other medications, including anti-histamines, were excluded during the study. All patients underwent skin tests for allergies and had their blood tested for IgE antibodies; with all results positive in the study group and negative in the control group. All participants gave written informed consent, and our study was approved by the local Ethics Committee.

2.2. Chemicals

Fexofenadine (FXF; PubChem CID: 63002), osthole (PubChem CID: 10228) and histamine (PubChem CID: 774) were obtained from Sigma-Aldrich, and all were prepared as in Kordulewska et al. (2015).

2.3. PBMC isolation

Subject blood was collected in K_3 ETDA tubes (BD, Biosciences) and PBMC isolation began immediately. Cell collection by Histopaque reagent (Sigma) was as in Kordulewska et al. (2015). PBMC's were counted by Scepter automatic cell counter (Merck Millipore) and seeded for up to 3 days in 24-well plates in 1×10^6 /0.5 ml of RPMI-1640 (Sigma) containing 1% gentamicin, 1% human AB serum, and 0.25% phytohaemagglutinin (PHA, Roche); at 37 °C in humidified 5% CO₂. PBMC's were in medium alone or with histamine, FXF and osthole in concentrations of 150 ng/ml histamine and 300 ng/ml FXF and osthole. These concentrations were chosen because this is the FXF human serum level following its administration; and similar osthole concentrations were required for comparison. The PBMC suspension was then centrifuged at 800g and 20 °C for 5 min and the cell residue was rinsed twice with Dulbecco's phosphate-buffered saline (DPBS, Invitrogen). The supernatant and plasma were collected and stored at -80 °C for further analysis.

2.4. Cytokine determination

Commercial ELISA Diaclone kits determined interleukin IL-1 β , IL-

10 and tumor necrosis factor (TNF- α) output and Mabtech kits determined interleukin IL-4 and IL-13, via quantitative sandwich immunoassay; with kits used according to manufacturer instructions. Triplicate samples were run and the results were equalized by comparison with standard curves expressed in pg/ml.

2.5. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Results are presented as mean $\pm$ S.E.M., and mean values between the control and allergic groups were compared using:

1. ANOVA test ($P < 0.0001$) for cytokine secretion in PBMC
2. unpaired t test ($P < 0.0001$) with equal S.D. for IgE and cytokine secretion in plasma
3. Pearson's correlation ($P < 0.0001$) correlation between IgE and cytokine secretion in plasma.

3. Results

The influence of the above active substances on cytokine secretion in PBMC's and serum was assessed: cytokines were IL-1 β , IL-4, IL-10, IL-13 and TNF- α , and cytokine levels secreted by untreated PBMC's in pure culture medium formed the absolute control.(ctrl).

3.1. Secretion of cytokines in PBMC

3.1.1. IL-1 β secretion

The lowest levels of IL-1 β with all examined substances were observed in the controls (Fig. 1A). PBMC incubation with histamine had the greatest IL-1 β increases in the control and study groups (Fig. 1A and B). In contrast, PBMC incubation with FXF had the lowest IL-1 β in the control group (Fig. 1A and B - significance in control vs. study group $P=0.0006$). This same result was observed in cells cultured in the pure medium control; where osthole, histamine/FXF and histamine/osthole in the control vs. study group, ctrl in control vs. study group indicated significantly increased $P < 0.0001$, $P=0.0001$, $P=0.0002$ and $P=0.0001$; respectively).

No statistically significant differences in examined substances were established in the control and study groups (Fig. 1B); and no statistically significant differences between histamine/FXF vs. histamine/osthole were determined (Fig. 1C).

3.1.2. IL-4 secretion

Histamine, FXF and osthole caused statistically significant increase in PBMC IL-4 secretion in the study group compared to the control group (Fig. 2A). While no statistical significance was observed in IL-4 secretion in the control group (Fig. 2B), histamine had significantly increased study group IL-4 compared to controls (Fig. 2B). FXF and osthole significantly decreased IL-4 secretion in cultured cells compared to histamine ($P < 0.01$ and $P < 0.001$, respectively), and we also observed a similar result in combined histamine/osthole treatment where cells decreased IL-4 secretion compared to histamine ($P < 0.01$) (Fig. 2B and C).

3.1.3. IL-10 secretion

All examined substances caused statistically significant increase in PBMC IL-10 secretion in the study group compared to the control group ($P < 0.0001$ - Fig. 3A). PBMC incubation with osthole had the greatest IL-10 levels in the study group, compared to histamine which had the lowest at $P < 0.01$ (Fig. 3A and B). While no statistical differences were noted in the tested control group substances (Fig. 3B), histamine effect was noticeable in the study group but not in the pure medium control. Histamine statistically depressed the level of IL-10 (ctrl vs. histamine $P=0.0003$), but PBMC's cultured with FXF

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