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Changes in gene expression induced by histamine, fexofenadine and osthole: Expression of histamine H₁ receptor, COX-2, NF- κ B, CCR1, chemokine CCL5/RANTES and interleukin-1 β in PBMC allergic and non-allergic patients

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ABSTRACT

Introduction: Fexofenadine (FXF) is a third-generation antihistamine drug and osthole is assumed as a natural antihistamine alternative. This paper compares results of histamine, FXF and osthole impact on HRH-1, COX-2, NF- κ B-p50, CCR1 mRNA expression. We also measured mRNA expression of IL-1 β and CCL5/RANTES in incubated peripheral blood mononuclear cells (PBMC) to compared how histamine, FXF and osthole had influence on expression level and interacts on product secretion.

Objective: The purpose was to investigate expression pattern in asthma PBMC.

Methods: The cultures were treated 72 h with FXF and osthole. We measured mRNA expression of histamine HRH-1, COX-2, NF- κ B-p50, CCR1, IL-1 β and CCL5/RANTES with Real-Time PCR (RT-PCR).

Results: The present study suggest that osthole may be a potential inhibitor of histamine H₁ receptor activity. We also demonstrated that cells cultured with histamine increase COX-2 mRNA expression and osthole reduce it.

Conclusion: Allergy remains one of the most common chronic diseases in Europe and it is rapidly approaching epidemic proportions; with current predictions estimating that the number of allergy-afflicted will equal the healthy population by 2020. It is therefore paramount to find new pharmaceuticals which successfully combat allergic disease.

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1. Introduction

Allergic inflammation is a chronic disease of the lower airways that affects millions of people worldwide. Allergic diseases are caused by hypersensitivity of the immune system to common environmental substances (Galli et al., 2008); where the immune response usually relies on the production of IgE antibodies specific to common allergens. The activation of mast cells and basophils through cell-surface-bound IgE then causes release of histamine and other mediators, leading to allergic inflammation (Douglass and O'Hehir, 2006; Hawrylowicz and O'garra, 2005; Thurmond

et al., 2008). Histamine is one of the major chemical mediators in allergic response pathophysiology (Slater et al., 1999). It is stored mainly in mast cells and basophilic granulocytes and is released by immunological or non-immunological stimuli. After release, histamine effects are mediated through action on multiple receptors, including histamine H₁, H₂, H₃ and H₄ (Yamaura et al., 2009). The biological effects of histamine on H₁ receptors include smooth muscle contraction, bronchospasm, increased vascular permeability and stimulation of sensory nerves and cough receptors (Jutel et al., 2001). The clinical consequences are manifested as urticaria, allergic rhino-conjunctivitis, broncho-constriction in asthma and cutaneous reactions to allergens and chemicals (Debacker et al., 1993; Kreutner et al., 2000; Matsubara et al., 2005).

Osthole has been used as an herbal drug in traditional medicines for thousands of years (Kermani et al., 2016) with immunomodulatory (You et al., 2009), anti-inflammatory (Asif, 2015; Chao et al., 2010), anti-tumor (Zhou and Liu, 2005) and anti-allergic effects (Kordulewska et al., 2015). The development of osthole and

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its derivatives as potential multi-target benefits should be encouraged because of their reported multiple bioactivities. It is therefore important to summarize pharmacological and biological research on this coumarin and review the mechanisms inducing its effects to clarify its precise functions (Jiao et al., 2016). In addition, Simpson et al. (2000) report that fexofenadine (FXF), the active metabolite of terfenadine, has anti-inflammatory properties. FXF is a selective histamine H₁ receptor antagonist unable to cross the blood brain barrier (Simpson and Jarvis, 2000).

A series of genes are expressed in the allergic response to pro-inflammatory mediators. Here, we chose genes which are critical in allergic inflammation and contribute to allergy pathology.

One principal anti-inflammatory drugs mechanism relies on the inhibition of COX-2 which is the rate limiting enzyme in the arachidonic acid pathway. COX-2 is induced during inflammation and leads to formation of pathological prostaglandin levels. Regulation of COX-2 and NF- κ B gene expression is a complex process which varies in different species and cell types. COX-2 gene expression is induced by a wide variety of stimuli such as proinflammatory cytokines, growth factors, differentiation factors, endotoxins, tumor promoters, reactive oxygen intermediates and cell-cell interactions. The NF- κ B pathway is one of the critical pathways mobilized by interleukin IL-1 β to induce COX-2 expression in intestinal myofibroblasts.

While anti-inflammatory drugs inhibition of prostaglandin synthesis has been demonstrated to effectively reduce inflammatory symptoms (Manrique-Moreno et al., 2016), glucocorticoids are by far the most effective treatment currently available for asthma and allergic reactions because they reduce asthmatic and allergic airway inflammation by inhibiting expression of cytokines and other mediators in the airway epithelial cells. In addition, glucocorticoids directly inhibit activated NF- κ B by protein-protein interaction with glucocorticoid receptors and also inhibit COX-2 expression in epithelial cells; most likely *via* direct repression of NF- κ B (Barnes and Adcock, 1997).

CCR1 is a CC chemokine receptor which binds a variety of chemokine ligands, notably CCL3 and CCL5, both of which are found in allergen-challenges, including bronchio alveolar lavage fluid (BALF) from asthmatic lungs (Alam et al., 1996; Cruikshank et al., 1995) and allergic rhinitic nasal secretions (Kramer et al., 2001). CCR1 deficiency blockade in the murine model of allergic conjunctivitis reduces disease score following co-stimulation of CCR1; thus suggesting the potential success of anti-CCR1 therapeutics in allergies (Wells et al., 1998). Hence, we propose HRH-1, COX-2, NF- κ B and CCR1 are compelling targets in the development of new anti-inflammatory drugs for allergic patients.

Antioxidants can block NF- κ B activation in response to a wide variety of stimuli and drugs, including pyrrolidine dithiocarbamate, and although they have proved useful in *in vitro* studies, they have proven too toxic for *in vivo* development. Osthole is considered an appropriate alternative to antioxidants, because our previous findings in osthole's anti-inflammatory role in PBMC's revealed it is a natural histamine antagonist capable of beneficial use in anti-histamine treatment of allergic conditions. Osthole is a favorable therapeutic option because it lacks toxicity and bestows both direct and indirect positive effects on healthy cells (Kordulewska et al., 2015).

Unfortunately, however, the molecular mechanisms and gene expression of the main inflammatory genes remain unresolved. Therefore, we investigated the *in vitro* effects of FXF and osthole on human HRH-1, COX-2, NF- κ B, CCR1 and the formation of the chemokine CCL5/RANTES and pro-inflammatory cytokine IL-1 β . In addition to gaining insight into FXF and osthole action mechanisms, the importance of osthole mRNA gene expression in inflammatory processes demanded determination of the key signaling pathways that regulate gene expression in patients with diagnosed allergy.

PBMC's isolated from whole blood were used in our *in vitro* investigation to most closely duplicate *in vivo* conditions, and control group comparison determined the affected signal pathways.

2. Materials and methods

2.1. Study participants

Our study group comprised 30 individuals aged 25–40 years diagnosed with allergies (age: M = 31.10 years, SD = 4.94) and thirty age-matched apparently healthy people without allergies or infections formed the controls (age: M = 30.90 years, SD = 4.95). Subjects with fever, infections or skin problems, previous smokers and those taking steroids or antibiotics were excluded from the study group. All patients underwent skin tests for allergies and blood tests for IgE antibodies; where the subject group results were all positive and the control group's negative. All participants gave written informed consent and the study was conducted under approval of 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, ST. Lois, MO, USA. The FXF was dissolved in 8% dimethylsulfoxide (DMSO, Sigma-Aldrich, ST. Lois, MO, USA) to final concentration below 0.1%; thus not affecting cell viability. The osthole was dissolved in 96% ethyl alcohol (AbChem, Poland) and the histamine in RPMI-1640 (Sigma-Aldrich, ST. Lois, MO, USA). All solutions were sterilized through a 0.22 μ m filter and stored at 4 °C as stock solutions for later dilution.

2.3. PBMC isolation

Peripheral blood samples were collected from participants between 7.00 and 9.00 a.m. on the first study day and these were concentrated in grouping tubes with K₃EDTA (Grainer Bio-One, GmbH, Austria). Fresh PBMCs were then prepared as previously described (Kordulewska et al., 2015). Isolated cells were seeded on 24-well plates (Sarstedt, Germany) at 1×10^6 per well with RPMI-1640 and supplemented with 1% heat inactivated human AB serum, 1% gentamicin and 0.25% PHA. Active reagents were added to each well after 24 h and pure medium formed the control for each substance. Cells were then harvested after a further three days.

2.4. Isolation of total RNA

Cells were collected by centrifugation at 800 $\times$ g for 10 min at 20 °C (Eppendorf Centrifuge 5804R, Germany) and lysed in 1 ml TRIzol Reagent (Life Technologies, UK) by repetitive pipetting. After incubation of the samples for 5 min at room temperature to allow complete dissociation of nucleoprotein complexes, 0.2 ml chloroform (ABChem, Poland) was added per 1 ml of TRIzol Reagent. The samples were mixed vigorously and centrifuged at 12,000g for 15 min at 4 °C. Centrifugation separated the biphasic mixtures into the lower red, phenol-chloroform phase and the upper colorless aqueous phase.

RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropanol. The samples were incubated at room temperature for 10 min and centrifuged at 12,000g for 10 min at 4 °C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol. The pellet was then air dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water. RNA purity was estimated by calculating the ratio between absorbance at 260 and 280 nm (A_{260}/A_{280}), with 1.8–2.0 result, and stored at –80 °C for

further analysis. We then determined DNA quality by agarose gel electrophoresis run as a single band.

2.5. Reverse transcription

Purified RNA (50–200 ng) was reverse transcribed by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, UK) according to manufacturer instructions and templates were store at -20°C in RNase-free water for further analysis.

2.6. Quantitative real time RT-PCR (qRT-PCR) and data analysis

Conditions for all RT-PCR analysis were optimized at $55\text{--}65^{\circ}\text{C}$ melting point and primer concentrations before commencing relevant experiments.

Seven genes: HRH-1, COX-2, NF- κ B-p50, CCR1, IL-1 β , CCL5/RANTES and the housekeeping human β -actin gene (ACTB) were examined; with ACTB used as reference gene to normalize differences in total RNA amounts in each sample. Oligonucleotide primers specific to each gene were designed with Primer-BLAST, and the PCR primers are listed in Table 1.

RT-PCR was performed in the LightCycler 96 Real-Time PCR System with the FastStart Essential DNA Green Master Kit (both Roche Diagnostics, Switzerland). The reaction was performed in triplicate in a total volume of $20\text{ }\mu\text{l}$ containing: $10\text{ }\mu\text{l}$ Master Mix, $5\text{ }\mu\text{l}$ cDNA template, $2\text{ }\mu\text{l}$ of each primer and $3\text{ }\mu\text{l}$ PCR-grade water per well. The following LightCycler experimental-run protocol was used: denaturation programme (95°C for 10 min), amplification and quantification program repeated 45 times (95°C for 20 s, $60/62/63^{\circ}\text{C}$ for 20 s, and 72°C for 20 s with a single fluorescence measurement), melting curve program $60\text{--}95^{\circ}\text{C}$ with 0.1°C per second heating rate and continuous fluorescence measurement and final cooling to 4°C . A negative control without cDNA was included in each assay and amplification product melting curve analysis was performed at the end of each PCR to confirm only one product was amplified and detected. The gene-expression relative quantification followed Pfaffl (2001) and the normal bladder sample with median ACTB expression was designated comparator.

2.7. Statistical analysis

All statistical analyses were performed in triplicate by GraphPad Prism version 6.0 (GraphPad Software, Inc., USA). The statistical significance of difference between mean values $\pm$ standard error was determined by analysis of variance (ANOVA); with $p < 0.01$ (99% confidence interval).

3. Results

Concentrations of 300 ng/ml FXF and osthol and 150 ng/ml histamine were evaluated to determine changes in PBMC mRNA expression in both healthy and allergic groups in:

These concentrations were chosen because this is the FXF human serum level following its administration; and similar osthol concentrations were required for comparison.

3.1. Histamine H_1 receptor mRNA expression results

The HRH-1 mRNA test effects on cultured cells are presented in Fig. 1. Sidak's multiple comparison test revealed statistical significance in the cells of both groups treated with histamine ($p = 0.01$); where HRH-1 mRNA expression was two and four times higher in the study and control groups, respectively. We also determined statistically significant difference in control vs. histamine and control vs. histamine/FXF in the study group at $p < 0.0001$ and

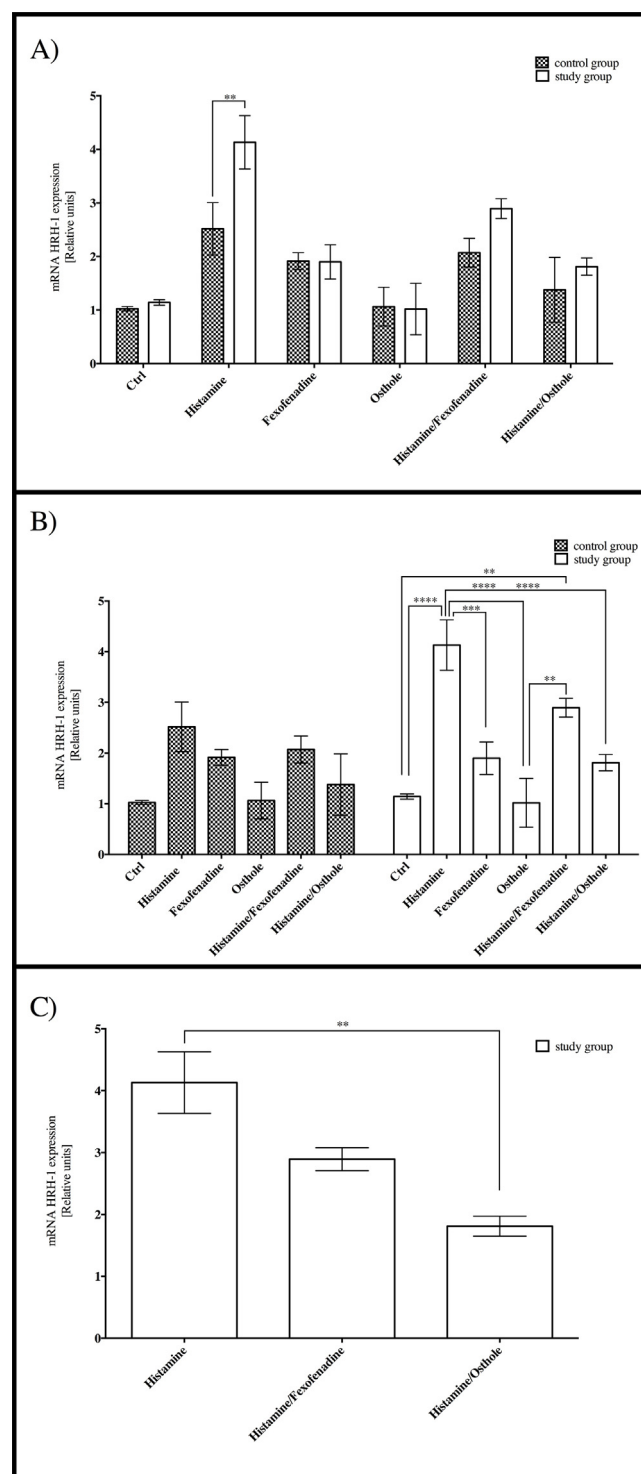


Fig. 1. Comparison of mRNA HRH-1 gene expression changes in PBMC in control and allergy groups under the influence of histamine, FXF, osthol, histamine/FXF and histamine/osthol. (A) between control and study group, (B) in the study group and control group, (C) histamine vs. histamine/FXF and histamine/osthol in the study group. Ctrl is HRH-1 gene expression in native cells; presented as 1. Statistically significant differences between the control and tested sample is shown directly above the error bar: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, $n = 30$.

$p < 0.01$ respectively. FXF ($p < 0.001$) and osthol ($p < 0.0001$) inhibited increased HRH-1 mRNA expression induced by histamine in the study group. This result was also observed in cells cultured with histamine/osthol; where combined substances decreased HRH-1 mRNA expression compared to histamine ($p < 0.0001$). This

Table 1

Primers used in PCR; with PCR primer nucleotide sequences used to assay gene expression in real-time quantitative PCR.

en	Forward primer	Revers primer	Primer depending temperature RT-PCR (annealing temperature)	base pairs
ACTB NM-001101.3	5'-TCC CTG GAG GAA GAG CTA CGA-3'	5'-AGC ACT GTG TTG GCG TAC G-3'	60 °C	194 bp
HRH-1 MN.001098213.1	5'-GCC GAG AGG ACA AGT GTG A-3'	5'-GGA GAC TCC TTC CCT GGT TT-3'	63 °C	244 bp
COX-2 M90100.1	5'-TGG CTA CAA AAG CTG GGA AG-3'	5'-ACT CAC CTT TGA CAC CCA AG-3'	63 °C	110 bp
NF-κB M58603.1	5'-TGG AGT CTG GGA AGG ATT TG-3'	5'-CGA AGC TGG ACA AAC ACA GA-3'	62 °C	129 bp
IL-1β NM.000576	5'-AAG GCG GCC AGG ATA TAA CT-3'	5'-CCC TAG GGA TTG AGT CCA CA-3'	61 °C	102 bp
CCR1 NM.001295.2	5'-CCA GTG ATC TAC GCC TTC GT-3'	5'-AGA GGA AGG GGA GCC ATT TA-3'	62 °C	103 bp
CCL5/RANTES DQ230537	5'-CGC TGT CAT CCT CAT TGC TA-3'	5'-ACA CAC TTG GCG GTT CTTC-3'	61 °C	196 bp

indicates similar roles for osthole and FFX in allergy; where no statistically significant differences were established between histamine/FFX and histamine/osthole in study and control groups.

3.2. COX-2 mRNA expression results

COX-2 mRNA test effects on cultured cells are presented in Fig. 2, with no statistically significant differences for all examined substances observed between the study and control groups. The lowest COX-2 mRNA expression was observed in control group cells treated with FFX and osthole and the highest was recorded in the histamine treatment; at $p < 0.001$ and $p < 0.01$ respectively. No changes were revealed in COX-2 mRNA expression after stimulation by the examined substances; study values remained higher than controls. While histamine increased COX-2 mRNA expression in the study group compared to controls ($p < 0.001$), FFX ($p < 0.001$) and osthole ($p < 0.001$) inhibited the increase in COX-2 mRNA expression induced by histamine. A similar result was observed in cells cultured with histamine/osthole; where combined substances decreased COX-2 mRNA expression compared to histamine at $p < 0.01$. Again, no statistically significant differences were established between histamine/FFX and histamine/osthole treatments.

3.3. NF-κB-p50 mRNA expression results

We investigated NF-κB-p50 after stimulation with the examined substances because it is one of the most important regulators of proinflammatory gene expression. Here, PBMC's exhibited the same tendency in both groups without statistical significance (Fig. 3). While lower mRNA expression level was observed in the study group after stimulation with FFX and their means were lower than control's. Study group cells treated with histamine had higher mRNA expression. Meanwhile, incubation with histamine in the control group induced statistically significant increased mRNA expression compared to FFX at $p < 0.0001$ and osthole at $p < 0.001$; and similar difference was noted in cells incubated with FFX and osthole in the study group where both substances decreased NF-κB-p50 mRNA expression induced by histamine ($p < 0.0001$). The study group also had statistically higher mRNA expression than controls in cells cultured with histamine ($p < 0.001$), and no statistically significant differences were found in the study group between histamine/FFX and histamine/osthole treatments.

3.4. CCR1 mRNA expression results

While no statistically significant differences in CCR1 mRNA effects on cultured cells were recorded between study and

control groups in our Fig. 4, Sidak's multiple comparison test provided $p = 0.01$ statistical significance in the cells of both groups treated with histamine. Our study group cells cultured with histamine revealed statistical increase over FFX and osthole at $p < 0.0001$; and while further statistical significant difference was noted in the control vs. histamine groups at $p < 0.0001$, once again our study group recorded no statistically significant difference in histamine/FFX and histamine/osthole treatments.

3.5. IL-1β mRNA expression results

The IL-1β mRNA effects on cultured cells in Fig. 5 reveal significant difference between control and study groups only in cells cultured with histamine ($p < 0.0001$). Study of substances added to controls showed that while combined histamine/FFX and histamine/osthole statistically increased IL-1β mRNA expression compared to the control group ($p < 0.0001$) and also increased it compared to FFX and osthole individually ($p < 0.0001$), both FFX and osthole decreased expression compared to histamine ($p < 0.0001$). Meanwhile, study of differences between the study and control groups highlighted that combined histamine/FFX and histamine/osthole statistically increased IL-1β mRNA expression in the study group over controls ($p < 0.0001$) and over FFX and osthole individually ($p < 0.0001$), while histamine increased this expression compared to both FFX and osthole ($p < 0.0001$).

3.6. CCL5/RANTES mRNA expression results

CCL5/RANTES has long been recognized as crucial in allergy pathogenesis. Our CCL5/RANTES mRNA test effects on cultured cells are presented in Fig. 6; where we observed the following statistically significant differences between control and study groups in cells cultured with histamine ($p < 0.0001$), FFX ($p < 0.001$), histamine/FFX ($p < 0.0001$) and histamine/osthole ($p < 0.001$). Addition of reagents to the study group gave the following statistically significant differences in CCL5/RANTES mRNA expression (1) increased expression ctrl. vs.: histamine and FFX ($p < 0.0001$); osthole ($p < 0.001$) and histamine/FFX and histamine/osthole ($p < 0.0001$). (2) differences in reagent effect; osthole significantly decreased expression compared to FFX ($p < 0.001$), and histamine/FFX and histamine/osthole increased expression compared to individual FFX and osthole addition ($p < 0.0001$).

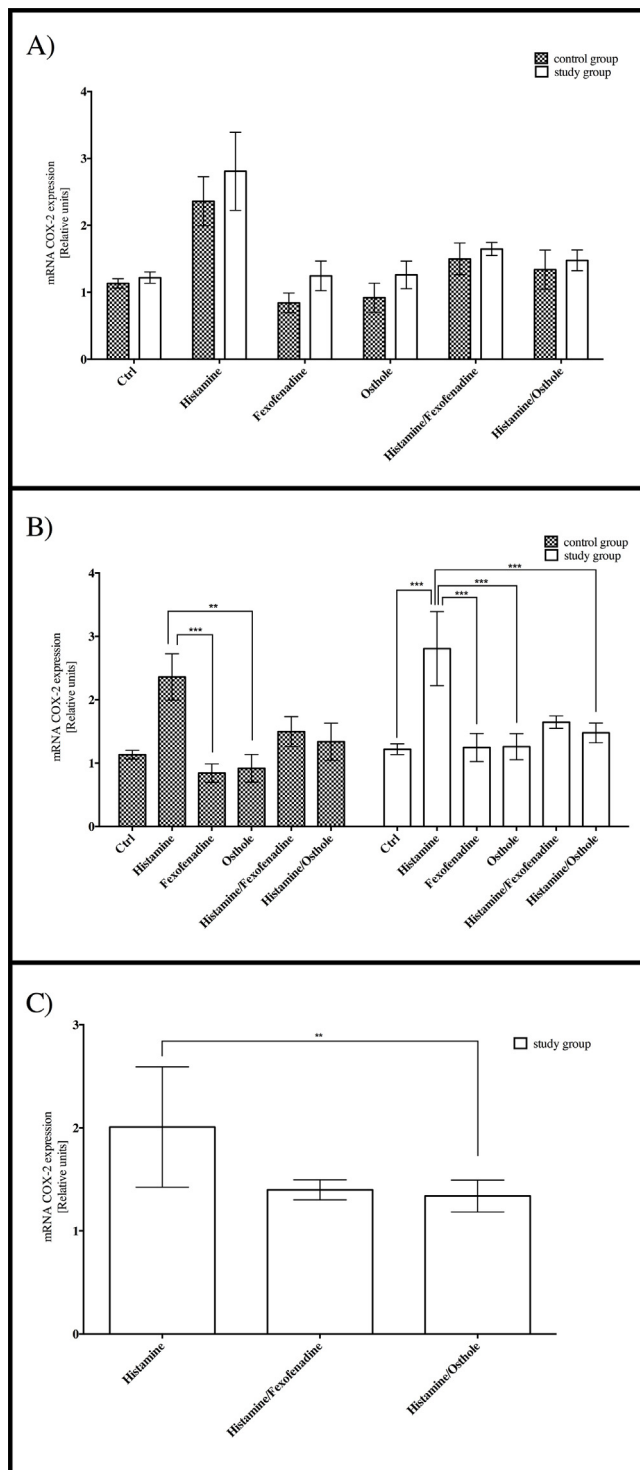


Fig. 2. Comparison of mRNA COX-2 gene expression changes in PBMC in control and allergy groups under the influence of histamine, FXF, osthole, histamine/FXF and histamine/osthole. (A) between control and study group, (B) in the study group and control group, (C) histamine vs. histamine/FXF and histamine/osthole in the study group. Ctrl is COX-2 gene expression in native cells; presented as 1. Statistically significant differences between the control and tested sample is shown directly above the error bar: **p < 0.01, *** p < 0.001, n = 30.

3.7. Pearson's correlation between histamine H_1 receptor, CCR1 and COX-2, NF- κ B-p50, IL-1 β and CCL5/RANTES

The correlations between receptors – HRH1 and CCR1 mRNA expression and COX-2, NF- κ B-p50, IL-1 β and CCL5/RANTES in

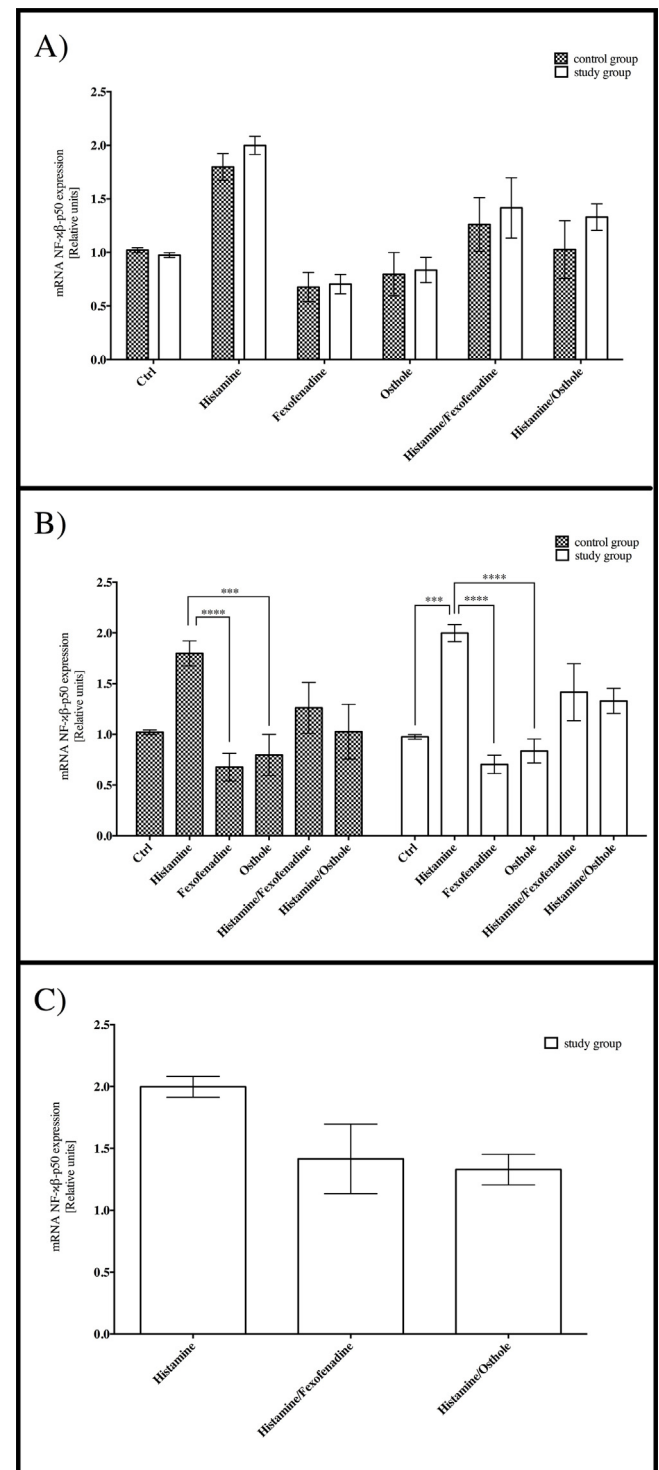


Fig. 3. Comparison of mRNA NF- κ B-p50 gene expression changes in PBMC in control and allergy groups under the influence of histamine, FXF, osthole, histamine/FXF and histamine/osthole. (A) between control and study group, (B) in the study group and control group, (C) histamine vs. histamine/FXF and histamine/osthole in the study group. Ctrl is NF- κ B gene expression in native cells; presented as 1. Statistically significant differences between the control and tested sample is shown directly above the error bar: ***p < 0.001, **** p < 0.0001, n = 30.

the control and study groups are presented in Table 2. Here, we observed almost complete correlation between HRH-1/COX-2 and HRH-1/IL-1 β in the study group. In similar investigation with CCR1/COX-2, CCR1/NF- κ B-p50 and CCR1/IL-1 β , the control group registered only the same correlation between CCR1 and CCL5 and

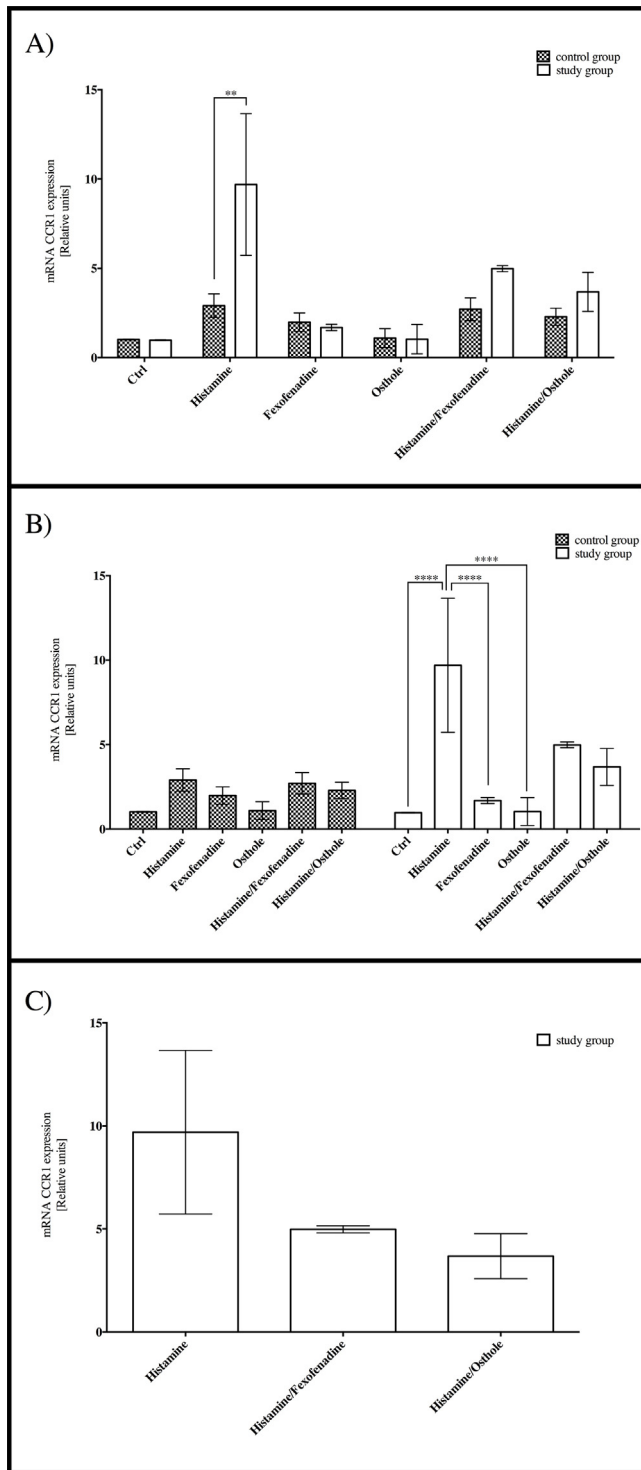


Fig. 4. Comparison of mRNA CCR1 gene expression changes in PBMC in control and allergy groups under the influence of histamine, FXF, osthole, histamine/FXF and histamine/osthole. (A) between control and study group, (B) in the study group and control group, (C) histamine vs. histamine/FXF and histamine/osthole in the study group. Ctrl is IL-1 β gene expression in native cells; presented as 1. Statistically significant differences between the control and tested sample is shown directly above the error bar: ****p < 0.0001, n = 30.

no further correlations were established between age or gender in either group. Our Figs. 7 and 8 indicate that increased expression of HRH-1 or the CCR1 receptor also increased the level of COX-2, NF- κ B-p50, IL-1 β and CCL5/RANTES mRNA. This result was reversed in PBMC's. These findings suggest that histamine, FXF and osthole act

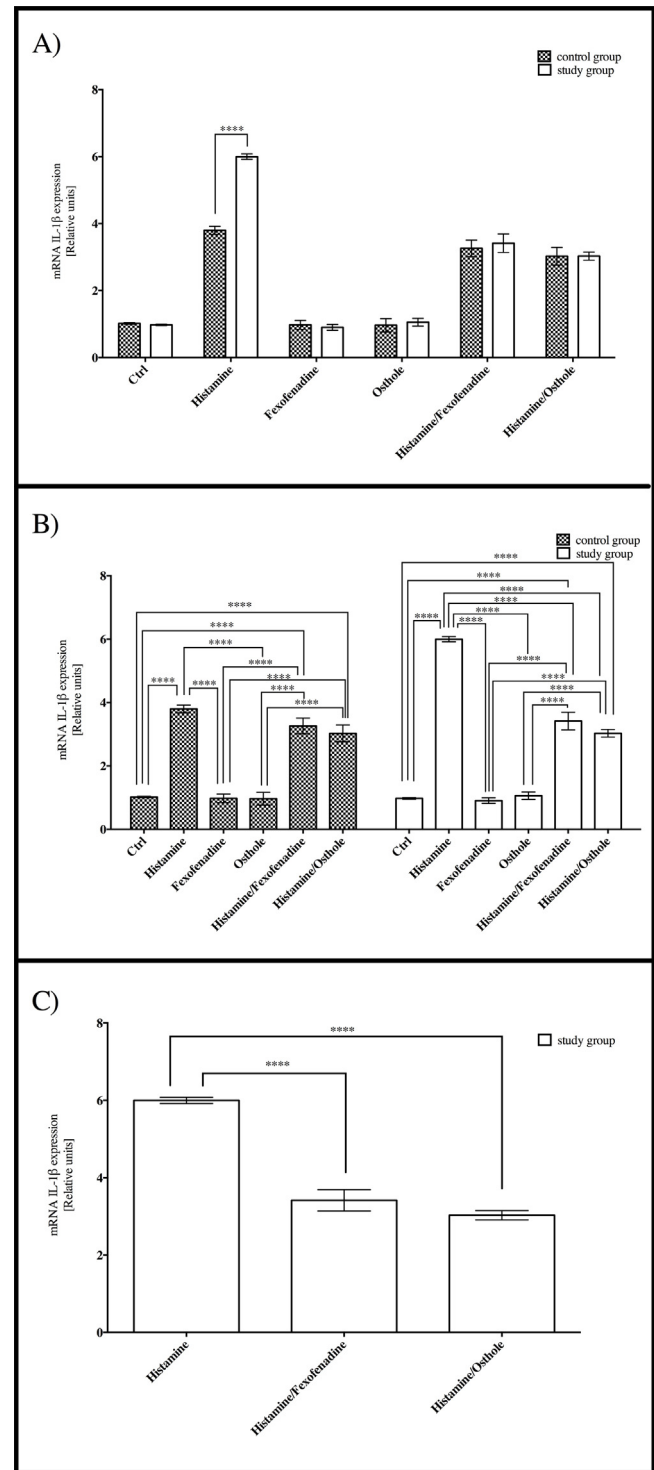


Fig. 5. Comparison of mRNA IL-1 β gene expression changes in PBMC in control and allergy groups under the influence of histamine, FXF, osthole, histamine/FXF and histamine/osthole. (A) between control and study group, (B) in the study group and control group, (C) histamine vs. histamine/FXF and histamine/osthole in the study group. Ctrl is CCR1 gene expression in native cells; presented as 1. Statistically significant differences between the control and tested sample is shown directly above the error bar: ****p < 0.0001, n = 30.

at induction and transcription levels for receptor HRH1 and CCR1 mRNA gene expression, in conjunction with COX-2, NF- κ B-p50, IL-1 β and CCL5/RANTES mRNA levels. It is most important here that both osthole and FXF reduced mRNA expression of all examined genes compared to histamine.

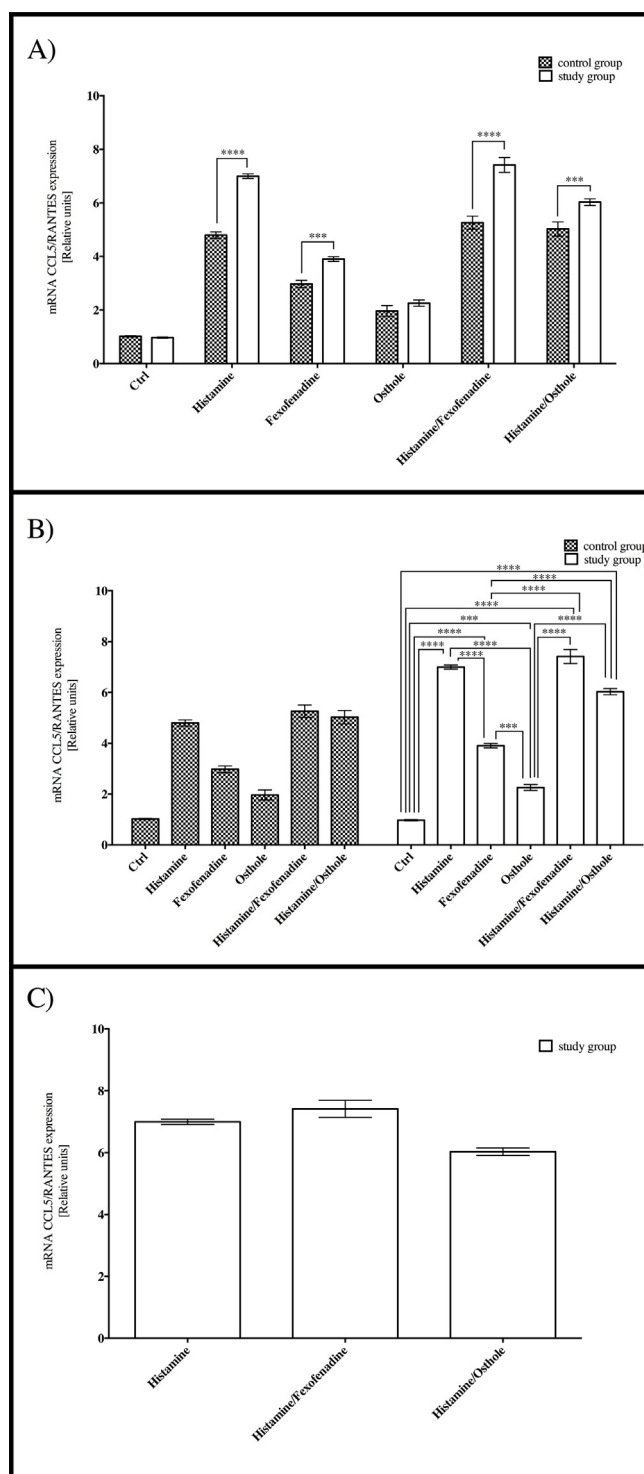


Fig. 6. Comparison of mRNA CCL5/RANTES gene expression changes in PBMC in control and allergy groups under the influence of histamine, FFX, osthole, histamine/FFX and histamine/osthole. (A) between control and study group, (B) in the study group and control group, (C) histamine vs. histamine/FFX and histamine/osthole in the study group. Ctrl is CCR1 gene expression in native cells; presented as 1. Statistically significant differences between the control and tested sample is shown directly above the error bar: ***p < 0.001, ****p < 0.0001, n = 30.

4. Discussion

Yamaura et al. (2009) investigated the effect of histamine H₁ receptor antagonist-resistant pruritus in a mouse model. The mice scratching behavior induced by 300 nmol histamine injected

Table 2

Pearson's correlation between the control and study group.

Control group – Pearson's correlation (R)	Study group – Pearson's correlation (R)
HRH-1/COX-2	HRH-1/COX-2
0.72	0.92
HRH-1/NF-κB-p50	HRH-1/NF-κB-p50
0.67	0.88
HRH-1/IL-1β	HRH-1/IL-1β
0.69	0.93
HRH-1/CCL5	HRH-1/CCL5
0.70	0.83
CCR1/COX-2	CCR1/COX-2
0.74	0.97
CCR1/NF-κB-p50	CCR1/NF-κB-p50
0.68	0.96
CCR1/IL-1β	CCR1/IL-1β
0.89	0.99
CCR1/CCL5	CCR1/CCL5
0.94	0.80

intradermally in the rostral region of their back was reduced by FFX treatment. We achieved the same result in our *in vitro* studies of histamine H₁ receptor expression. Although histamine statistically increased the histamine H₁ receptor expression level, combined histamine/FFX treatment reduced this mRNA expression level by half. This result confirms that FFX effectively blocks histamine H₁ receptor activity. It is highly important that we also established that osthole produced the same reduction in histamine H₁ receptor expression and that we recorded no statistical difference between the histamine/FFX and histamine/osthole effects in our *in vitro* studies (Fig. 1).

Further research by Giustizieri et al. (2004) examined the presence of functional histamine H₁ receptors on human keratinocytes and the capacity of histamine to modulate the expression of inflammatory molecules in these cells. Their cultured keratinocytes also constitutively expressed H₁ receptor mRNA after treatment with histamine, and they noted IFN-γ induced expression of membrane intercellular adhesion molecule 1 and MHC class I. It is interesting that similar to our study, they stimulated cells with a third-generation non-sedative antihistamine (levocetirizine) which is developed from the second-generation cetirizine. Levocetirizine administration abolished histamine-dependent expression of all investigated pro-inflammatory molecules in a dose-dependent manner.

While no available literature information exists on osthole activity in histamine H₁ receptor mRNA expression, our major objective in determining whether osthole has similar effect to FFX in blocking histamine action was successful, because we clearly confirmed that osthole and FFX both exerted the same reduction in histamine H₁ receptor expression.

COX is the rate-limiting enzyme in conversion of arachidonic acid to prostaglandin (PG) H₂; the precursor of various compounds including PGs, prostacyclin and thromboxanes which are important inflammatory mediators (Yang et al., 2011). At least two COX genes, COX-1 and COX-2, have been identified. COX-1 is constitutively expressed in many tissues and plays important roles in homeostasis. In contrast, COX-2 is an immediate early gene induced by various stimuli including mitogens, growth factors, cytokines, hormones and tumor promoters (Cheng et al., 2004). The COX pathway induces the production of prostaglandins through the constitutively expressed COX-1 and inducible COX-2 enzymes. COX-2 is upregulated in inflammation; therefore agents that suppress COX-2 activity may be potential drug targets to inhibit inflammation in allergies.

With respect to COX-2 gene transcriptional regulation, its promoter contains many *cis*-acting regulatory elements; of these only NF-κB site, CRE, NF-IL6 motif, and E-box are known to be involved

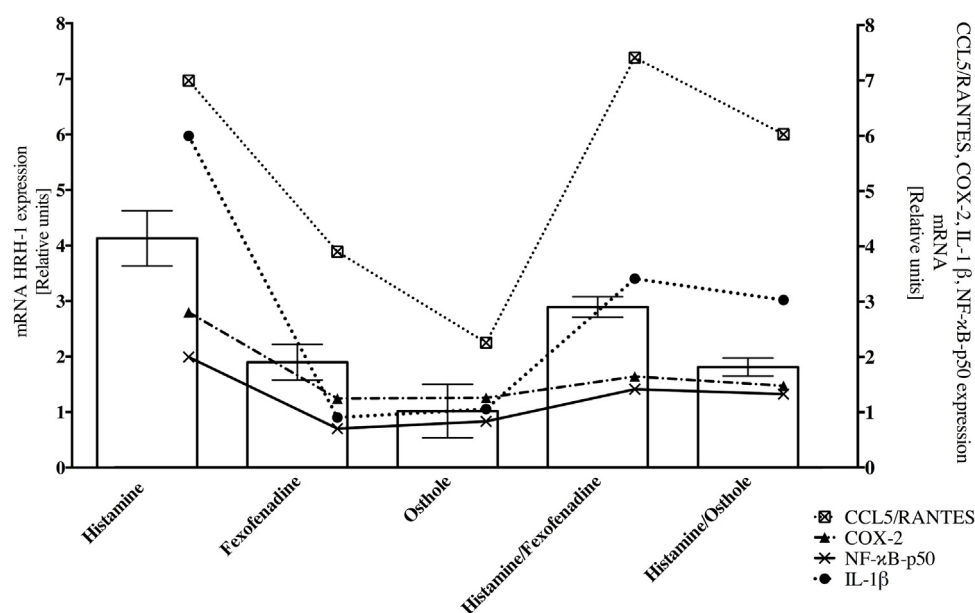


Fig. 7. Comparison of mRNA HRH-1 gene expression and protein mRNA CCL5/RANTES, COX-2, IL-1β, NF-κB-p50 gene expression changes in PBMC in allergy groups under the influence of histamine, FFX, osthole, histamine/FFX and histamine/osthole. Ctrl is gene expression in native cells; presented as 1.

in regulating COX-2 gene expression (Harper and Tyson-Capper, 2008; Thurmond et al., 2008). Transcription factors bind to these sites in a variety of combinations depending on cell type and the regulatory pathway activated. Our study established that histamine activates NF-κB transcription factor, and we speculate that histamine may have caused the marked NF-κB upregulation in our study, and also enhanced NF-κB binding to the COX-2 promoter; with COX-2 promoter activation leading to increased COX-2 mRNA expression.

We also demonstrated that cells cultured with histamine increase COX-2 mRNA expression and that FFX and osthole reduce it in both study groups (Fig. 2). It is interesting that mRNA COX-2 expression after stimulation is at a comparative level to that

in the control. Yang et al. (2001) reported similar results in polymorphonuclear neutrophil suppression of COX-2 mRNA expression when these cells were stimulated by a new mixed formula of Chinese herbs.

NF-κB is a major regulator of important cell processes, such as cellular proliferation, immunity, inflammation and apoptosis (Bakker et al., 2001; Gloire et al., 2006; Madonna and De Caterina, 2012; Barroso et al., 2016). It is part of the NF-κB family; a heterogeneous set of transcription factors that dimerize in different combinations at the common Rel homology domain. Five mammalian NF-κB proteins have been identified, including p65 (also called RelA), RelB, c-Rel, p50 (or NF-κB1) and p52 (or NF-κB2). These share a conserved DNA-binding domain (Hayden and Ghosh,

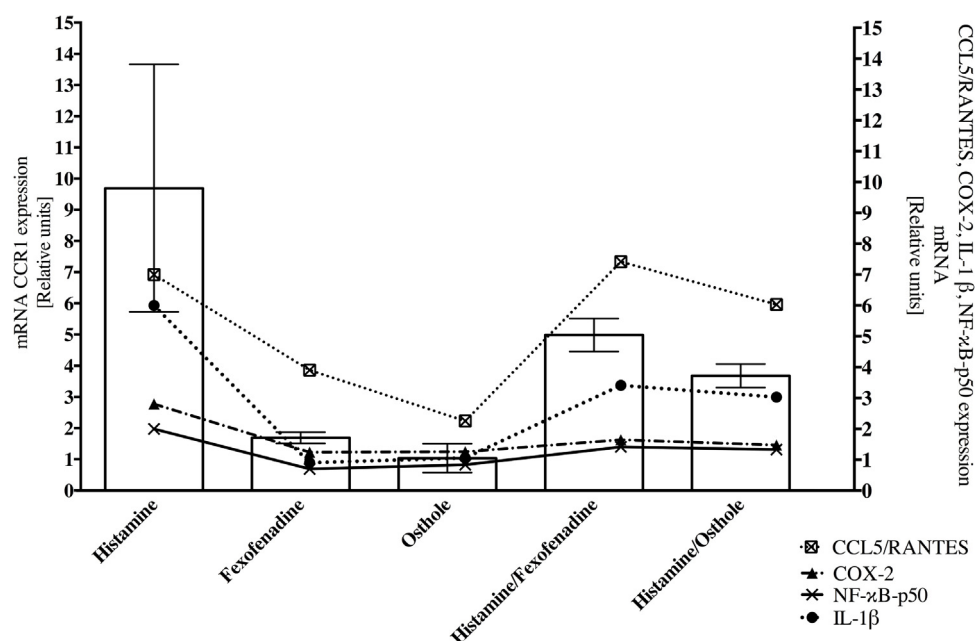


Fig. 8. Comparison of mRNA CCR1 gene expression and protein mRNA CCL5/RANTES, COX-2, IL-1β, NF-κB-p50 gene expression changes in PBMC in allergy groups under the influence of histamine, FFX, osthole, histamine/FFX and histamine/osthole. Ctrl is gene expression in native cells; presented as 1.

2004; Kumar et al., 2004). NF- κ B is important in regulating the transcriptional expression of many genes involved in inflammation and allergy dysfunction, and is therefore considered a promising therapeutic target to reduce allergic symptoms (Bakker et al., 2001; Hayden and Ghosh, 2004). This led us to examine NF- κ B in this study.

More recently, the H₁ receptor has also been shown to activate NF- κ B (Bakker et al., 2001; Aoki et al., 1998; Hu et al., 1999). Since both H₁ receptor and NF- κ B are involved in allergic conditions, it is attractive to speculate that coupling the H₁ receptor and NF- κ B pathways would be physiologically important in these disorders. Bakker et al. (2001) demonstrated that H₁ receptor mediated NF- κ B activation is inhibited by all clinically used H₁-antihistamines tested to date; including cetirizine, ebastine, epinastine, FFX, loratadine and mizolastine. This indicates all these drugs are inverse H₁-agonists. We obtained similar results; where histamine increased NF- κ B mRNA expression and FFX successfully inhibited it in our control. While expression was also inhibited by osthole, it is important that cells incubated with the combined histamine/FFX and histamine/osthole also inhibited NF- κ B mRNA expression (Fig. 3).

Hart et al. (1998) demonstrated increased NF- κ B activity in asthmatic patient airways and in inflammatory cells. They reported that cells from induced sputum and bronchial biopsies from mildly asthmatic patients increased NF- κ B expression. Our study also indicated higher NF- κ B mRNA expression in the study group than in controls, and that histamine's immunomodulatory effect increased NF- κ B expression in PBMCs. These findings suggest that NF- κ B has an important role in maintaining chronic inflammatory responses in allergies, because increased NF- κ B binding is associated with increased gene expression in relevant allergy cytokines and enzymes. Yamamoto and Gaynor (2001) and Christman et al. (2000) found that asthmatic pathogenesis involves persistent expression of pro-inflammatory cytokines, chemokines and other inflammatory mediators, and that the genes of many of these proteins contain NF- κ B binding sites in their promoters; suggesting NF- κ B plays a vital role in asthma.

NF- κ B is also implicated in inflammatory bowel disease; including Crohn's disease and ulcerative colitis (Neurath et al., 1998; Schreiber et al., 1998). NF- κ B activation is evident in biopsies from these patients, and treatment with steroids decreases NF- κ B activity in biopsies and reduces clinical symptoms. Furthermore, NF- κ B is involved in the pathophysiology of rheumatoid arthritis autoimmune disorder (RA). NF- κ B is upregulated there, and cytokines such as TNF- α which activate NF- κ B are elevated in the synovial fluid of RA patients (Feldmann et al., 1996; Roman-Blas and Jimenez, 2006). In addition to the roles that NF- κ B plays in inflammatory diseases, constitutive activation of the NF- κ B pathway is involved in cancers; including leukemia, lymphoma and colon and ovarian cancer (Rayet and Gelinas, 1999). Mutations which can lead to such tumors include inactivation of I κ B proteins and amplifications and rearrangements of genes encoding NF- κ B transcription factor subunits. However, it is more commonly considered that changes in upstream pathways leading to NF- κ B activation become deregulated in cancer.

Important features of our study are the clear demonstration that FFX and osthole significantly reduce NF- κ B expression (Fig. 3) and that these additionally examined substances have more influence on NF- κ B expression than histamine.

The pharmacological targeting of chemokines and chemokine receptors has long been recognized as a viable approach in treating allergy and asthma (Murdoch and Finn, 2000; Lloyd and Rankin, 2003; Proudfoot et al., 2003). Blease et al.'s. (2000) studies highlighted the importance of CCR1 in the persistence of airway remodeling during chronic fungal asthma, and these findings provided the impetus to explore whether the pharmacological

targeting of this chemokine receptor was as effective in attenuating this and other features of chronic allergic airway disease due to *Aspergillus*. It was observed that the systemic administration of the CCR1 antagonist with BX-471 anti-inflammatory activity beginning at day 15 after *Conidia* challenge in *A. fumigatus*-sensitized-mice attenuated all the features of airway disease typically observed in this model at day 30; including peribronchial inflammation and airway hyper-response, and remodeling. The effect of BX-471 on CC and CXCR chemokine receptor transcript expression, all of which have been implicated in allergic airway disease and asthma (Nagase et al., 2001), increases the therapeutic potential of CCR1 antagonism in clinical disease.

Similar data from our study shows that CCL5/RANTES and CCR1 were expressed by PBMC and that histamine increased CCR1 mRNA study-group expression compared to the control (Figs. 4 and 6). This suggests that CCL5/RANTES and CCR1 are involved in the accumulation of inflammatory cells in allergic inflammation. The regulation of CCR1 expression remains uncertain, although lipopolysaccharides, IFN- γ , IL-2 and IL-15 are known to induce CCR1 expression in monocytes, neutrophils and activated T cells (Schall et al., 1990). Our research confirmed that histamine induced CCR1 mRNA expression. We demonstrated almost complete correlation between CCR1 and COX-2, NF- κ B-p50 and IL-1 β , and also that FFX and osthole significantly decreased CCR1 mRNA expression compared to histamine. This latter finding supports osthole's anti-inflammatory properties.

The following results have also emerged from CCR1 research: (1) Lukacs et al. (2005) and Schwarze et al. (1997) elucidated the role of CCR1 and its ligand CCL5/RANTES in a model of respiratory syncytial virus (RSV) which developed before later cockroach allergen (CRA) sensitization and challenge; while previous studies had shown that RSV infection occurring before or during allergen sensitization could exacerbate the allergic response: (2) John et al. (unpublished data, 2004) found that CCR1/mice did not differ from their wild-type counterparts in airway hyper-response or inflammation in a CRA-induced asthma model (Miller and Lukacs, 2004). This result was similar to findings in a fungal-induced asthma model (Blease et al., 2000); (3) John et al. (2003) observed that CCR1/mice exhibited significantly decreased airway hyper-response and mucus overproduction over wild-type mice in a combined RSV–CRA model. These pathophysiological changes correlated with decreased levels of IL-4, IL-13, neutrophils, and CD4+ T cells. In addition, although CCL5 RSV induction is considered to contribute to exacerbated allergic response, its role appears inflammatory rather than pathophysiological. This combined data supports CCR1 as a viable target in treatment of long-term chronic asthma; especially asthma associated with viral and fungal infections.

IL-1 β is considered a major proinflammatory cytokine with pleiotropic roles in host defense, inducing acute and chronic inflammation through activation of both innate and acquired immune systems. IL-1 β research includes; (1) despite reports that IL-1 and caspase-1 mRNA levels produced by monocytes in patients with atopic dermatitis (AD) are lower than those in healthy subjects (Räsänen et al., 1987; Kapp et al., 1988; Higashi et al., 2001), other research indicates the reverse situation (Thestrup-Pedersen et al., 1989). (2) Kordulewska et al. (2016) observed that PBMC's from allergic individuals produced 5-fold IL-1 β compared to healthy people; this was supported by serum analysis. (3) Our Fig. 4 shows, that cells treated with histamine significantly induce IL-1 β mRNA expression in the study group over controls: (4) Bochner et al. (1990) observed upregulated IL-1 β mRNA protein expression in atopic-dermatitis-patients' skin (AD) following epicutaneous application of ragweed or house dust mite antigen; this did not occur in healthy subjects; (5) keratinocyte-specific IL-4-overexpressing-Tg-mice spontaneously developed skin inflammation similar to human AD and had increased IL-1 β mRNA expression

in local inflammatory lesions (Chen et al., 2008); this supports consideration that IL-1 is involved in AD development; (6) asthmatic alveolar macrophages produced IL-1 in response to stimuli such as silica and LPS (Oghiso and Kubota, 1986); (7) IL-1 induces proinflammatory mediators, including TNF, IL-6 and IL-8 and adhesion molecules, in airway smooth muscle, epithelial cells and endothelial cells, and it also augments IgE-mediated mast cell activation, such as Th2 cytokine secretion (Iikura et al., 2007); thus suggesting IL-1 involvement in development of IgE-mediated allergic diseases such as allergic asthma; and this is further supported by IL-1 β and IL-1Ra genes-promoter-polymorphism associated with susceptibility to the disease (Cookson, 2002); (8) Serum IL-1 β is increased in atopic asthmatics compared to non-atopic asthmatics and chronic obstructive pulmonary disease patients (COPD) (Mahajan et al., 2008); (9) IL-1 β and/or IL-1Ra expression increased in asthmatic bronchial epithelium and macrophages (Murillo et al., 2003); (10) since IL-1 enhances the mast cell cytokine secretion described above, and also histamine release (Subramanian and Bray, 1987), IL-1 may be involved in food allergy induction; as seen in (11) where administration of rIL-1Ra to guinea pigs reduced the IgE production and anaphylaxis induced by cow milk (Theodorou et al., 1993) and finally (12) genome-wide association study suggests that IL-1 α , IL-1 β and IL-1Ra polymorphism in allergic rhinitis patients is associated with susceptibility to this disease (Joki-Erkilä et al., 2003).

These combined observations, supported by our results, contend that IL-1 contributes to the pathogenesis of allergy and asthma. However, elucidation of IL-1's precise role in development of these allergic diseases remains dependent on investigating IL-1-related gene-deficient mice.

In conclusion, allergy remains one of the most common chronic diseases in Europe and it is rapidly approaching epidemic proportions; with current predictions estimating that the number of allergy-afflicted will equal the healthy population by 2020. It is therefore paramount to find new pharmaceuticals which successfully combat allergic disease (Barroso et al. 2016; Roman-Blas and Jimenez, 2006).

Conflict of interest

The authors declare they have no competing interests.

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