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*Original Research*

# Histamine H4 Receptor mediates interleukin-8 and TNF-α release in human mast cells via multiple signaling pathways

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**Abstract:** Histamine, mainly produced by mast cells, is an important inflammatory mediator in immune response. Recently Histamine H4 Receptor (H4R) was also identified in mast cells, from which pro-inflammatory cytokines and chemokines are released. However, the mechanism of how H4R mediates these cytokines and chemokines release in mast cells was still unclear. To further explore the role of H4R in the immune inflammatory response in mast cells, we tested the release of inflammatory cytokine tumor necrosis factor-α (TNF-α), chemokine interleukin-8 (IL-8) and the relevant signaling pathways activated by H4R on LAD2 cells (a human mast cell line). We found that the release of IL-8 and TNF-α were blocked by inhibitors of PI3K, ERK and $Ca^{2+}$-Calcineurin-NFAT signaling pathways, while the release of these cytokines and chemokines were enhanced by the inhibitor of P38 signaling pathway. However, inhibitors of the JNK and NF-κB signaling pathways had little effect on the expression of the pro-inflammatory mediators. Moreover, activation of the H4R could induce phosphorylation of ERK, p38 and AKT in mast cells. In conclusion, we found that H4R mediates the release of inflammatory cytokine TNF-α and chemokine IL-8 in human mast cells via PI3K, $Ca^{2+}$-Calcineurin-NFAT and MAPKs signaling pathways.

***Key words:*** Histamine, Histamine H4 receptor, Mast cells, IL-8, TNF-α.

## Introduction

Histamine is a biogenic amine that plays an important role in many physiological processes (1). Histamine mediated multiple signaling pathways in inflammation and allergic responses via four G-protein-coupled receptors named H1R, H2R, H3R and H4R. H1R was a classical receptor that modulated inflammatory and allergic responses (2). However, the therapeutic inhibitors of H1R and H2R could not restrain all symptoms of inflammation and allergic diseases (3).

It was found that H4R was expressed in hematopoietic cells, and played an important regulatory role in the activation of mast cells, eosinophils, monocytes, dendritic cells, and T cells (4, 5). Mast cells activated by H4R can release lots of inflammatory mediators to trigger and amplify inflammatory cascade on chronic phase of immuno reactions. Because of its important roles in chronic inflammation and allergic symptoms, H4R was considered as a new therapeutic target for allergic inflammation diseases such as asthma and rhinitis (6-8).

Although researches on H4R signaling pathways have been mainly focused on cAMP and intracellular calcium levels, it was showed that Histamine H4 receptor activation enhances LPS-induced IL-6 production in mast cells via ERK and PI3K activation (9). What's more, H4R activation led to induction of AP-1 in both dendritic cells and T cells (10, 11). Therefore, these reports put some insights into the downstream signaling pathways of the H4R.

LAD cell lines remain dependent upon stem cell factors for survival and proliferation, which express intracytoplasmic histamine, tryptase and chymase. H4R, expressed by mast cells including LAD2 cells and primary cord blood derived CD34+ human mast cells, plays a primary role in the progression of allergic inflammatory responses by enhancing the secretion of two important contributors, pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) and chemokine interleukin-8 (IL-8) (12-14). However, the relationship between these inflammatory protein release and the signaling pathways downstream of the H4R in mast cell remained unclear. In order to understand the mechanism of H4R on the activation of mast cells in allergic inflammation, we elucidated the H4R mediated functional activation of IL-8 and TNF-α in the human mast cells line LAD2 cells and related signal transduction pathways. Finally, it was found that H4R mediated the release of inflammatory cytokine TNF-α and chemokine IL-8 in human mast cells via PI3K, $Ca^{2+}$-Calcineurin-NFAT and MAPKs signaling pathways.

## Materials and Methods

### Cell Culture and Reagents

The Laboratory of Allergic Disease 2 (LAD2) human

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mast cells were kindly provided by A.Kirshenbaum and D.Metcalfe (NIH, USA) (15). Cells were maintained in StemPro-34 (Invitrogen, USA) medium as previously described (16). Clobenpropit was purchased from Sigma. PD 98059, SP 600125, SB 203580, Bay 11-7821, Ciclosporin were purchased from Tocris. Wortmannin was purchased from Cayman. When chemicals were dissolved in DMSO, the final concentration of DMSO did not alter the normal response of LAD2 cells.

### IL-8 and TNF-α measurement

After pre-incubated with various inhibitors for the corresponding time periods, LAD2 cells were incubated with different concentrations ($4\times10^{-5}$ M and $8\times10^{-5}$ M ) of clobenpropit for overnight. The release of IL-8 and TNF-α in the supernatants were measured by ELISA assay (BD Biosciences, USA) according to the manufacturer's instructions. The working concentrations of six inhibitors were determined as follows: PD 98059, 10μM; SP 600125, 20μM; SB 203580, 10μM; Bay 11-7821, 10μM; Ciclosporin, 2μg/ml; Wortmannin, 0.5μM.

### Western blotting

Whole cell lysate of The LAD2 cells was in RIPA buffer, while nuclear and cytoplasmic proteins were extracted by Nuclear and Cytoplasmic Protein Extraction Kit (Sangon, China). The following steps of Western blotting were performed as previously described (17). The primary antibodies were as following: Anti-Akt (cell signaling, Beverly, MA); Anti-phospho-Akt (Ser 473, cell signaling, Beverly, MA); Anti-JNK (cell signaling, Beverly, MA); Anti-phospho-JNK (Thr 183/ Tyr 185, cell signaling, Beverly, MA); Anti-ERK (cell signaling, Beverly, MA); Anti- phospho-ERK (Thr 202/ Tyr 204, cell signaling, Beverly, MA); Anti-p38 MAPK (cell signaling, Beverly, MA); Anti-phospho- p38 MAPK (Thr 180/ Thr 182, cell signaling, Beverly, MA); Anti-NFAT (cell signaling, Beverly, MA), Anti-NF-Kb (cell signaling, Beverly, MA), Anti-LaminB (Abcam, USA), Anti-β-tubulin (ProteinTech, China) .Then a secondary antibody (1:1000 diluted) (MBL) was added on the membrane for 1 h at room temperature. After incubated in ECL solution (Pierce Chemical, Rockford, IL), levels of proteins were detected and analyzed by a LAS-1000 Pro image reader.

### Statistical analysis

Statistical significance was determined by student t-test and one-way ANOVA. Significant differences were considered when P value was less than 0.05. All of the data were expressed as Means ±SEMs.

## Results

### H4R agonist induced IL-8 and TNF-α production in human mast cells

Mast cells have the ability to release various bioactive mediators such as cytokines and chemokines. Because IL-8 and TNF-α are considered as primary mediators of inflammatory responses, our effects on IL-8 and TNF-α production via H4R on mast cells were explored to further understand the mechanism of pro-inflammatory proteins release via H4R activation. Clobenpropit, one of H4R agonists, dose dependently induced IL-8 and TNF-α production in LAD2 cells (Fig.1A and Fig.1B). It suggested that H4R mediated the release of IL-8 and TNF-α in human mast cells.

### IL-8 and TNF-α release induced by H4R from activated LAD2 cells was inhibited by blockers of PI3K and $Ca^{2+}$-Calcineurin-NFAT signaling pathways

To find out the downstream signaling pathways of H4R activation which induce the release of IL-8 and TNF-α, LAD2 cells were treated with clobenpropit after pre-incubated with different signaling pathway inhibitors for 30 min.

Activation of calcineurin contributes to the release of IL-8 and TNF-α (18, 19). As shown in Fig. 2A, IL-8 and TNF-α release induced by clobenpropit were dramatically blocked by ciclosporin, which is the specific inhibitor of calcineurin. PI3K is well known for its role in human mast cells activity in response to various cell surface receptors including H4R (20). Consistent with previous reports, we found wortmannin, the PI3K inhibitor, inhibited IL-8 and TNF-α release induced by clobenpropit (Fig. 2B). NF-κB was considered to participate in the production of cytokines and chemokines driven by histamine from activated microglia (21). However, the release of IL-8 and TNF-α induced via clobenpropit were not reduced after the addition of the NF-κB inhibitor Bay 11-7821 (Fig. 2C).

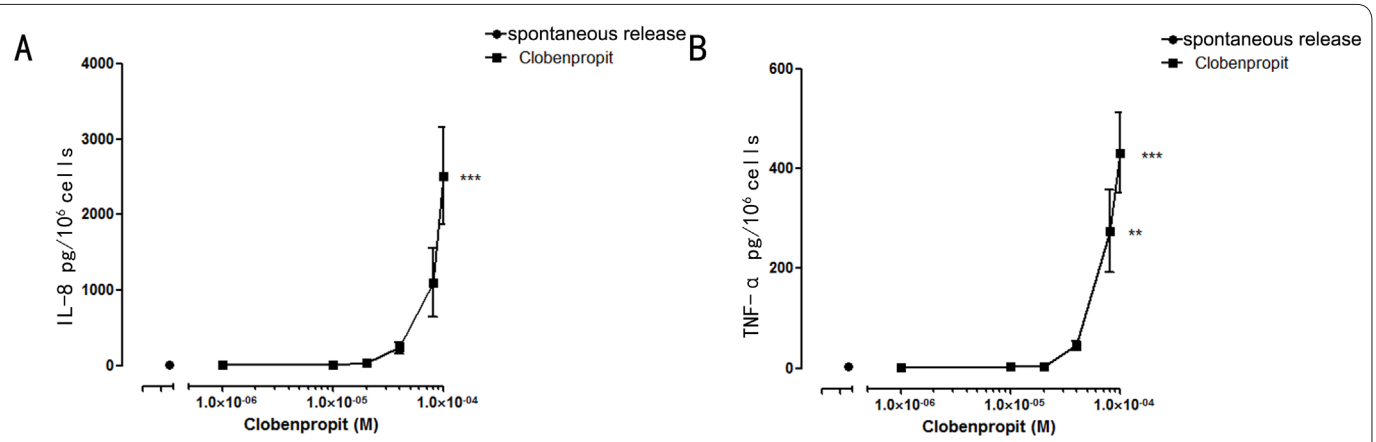


**Figure 1.** Effect of H4R agonist on IL-8 and TNF-α release from LAD2 cells. (A) The amount of IL-8 release from LAD2 cells incubated with the different concentrations of clobenpropit. (B) The amount of TNF-α release from LAD2 cells incubated with the different concentrations of clobenpropit. The levels of IL-8 and TNF-α spontaneous release and those in the present of H4R agonist were compared with one-way ANOVA and student t-test. *p<0.05, **p<0.01, ***p<0.001 (n = 3–5).

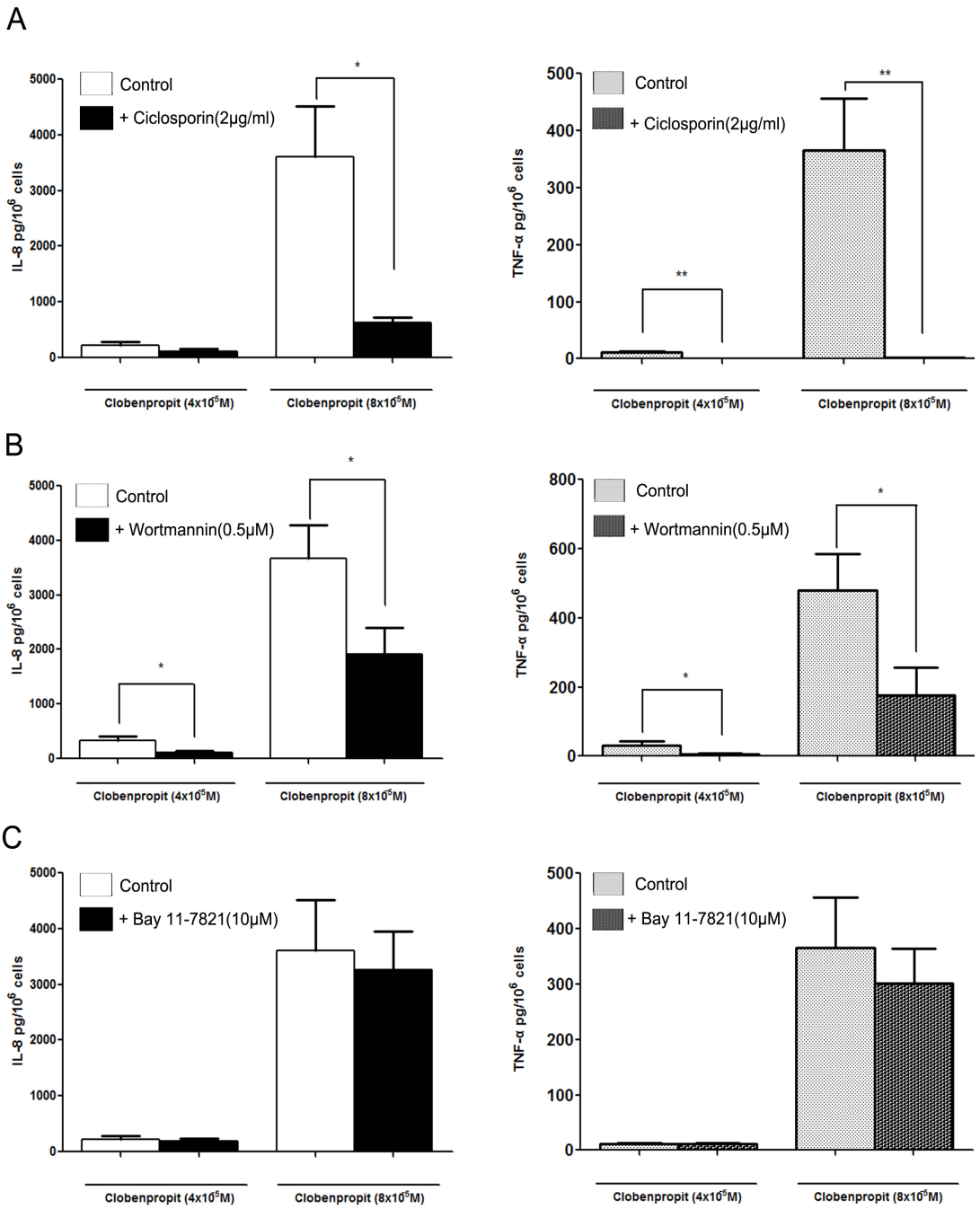


**Figure 2.** Effects of inhibitors of calcineurin, PI3K and NF-κB on H4R agonist induced IL-8 and TNF-α release from LAD2 cells. (A) ciclosporin (2 μg/ml), (B) wortmannin (0.5 μM) or (C) Bay 11-7821 (10 μM) was incubated with LAD2 cells for 30 min before the addition of two concentration of clobenpropit ($4\times10^{-5}$ M and $8\times10^{-5}$ M ) for 24 h to induce the release of IL-8 and TNF-α. The amounts of IL-8 and TNF-α release from activated LAD2 cells pre-incubated with an inhibitor and the corresponding control pre-incubated in culture medium were compared with student t-test. *p<0.05, **p<0.01 (n = 4–5).

## Role of MAPKs signaling pathways in the production of H4R induced IL-8 and TNF-α

MAPKs, of which the tyrosine phosphorylation could be induced by H4R, had been considered as important signaling molecules taking part in the release of various cytokines and chemokines in mast cells (14). Since ERK pathways are involved in inflammatory cytokines and chemokines release, inhibitors of ERK pathways were used for effects on the H4R-induced IL-8 and TNF-α production. Inhibitor of ERK (PD98059) was able to block the IL-8 and TNF-α response, located downstream of H4R activation (Fig. 3A). The role of p38 in the clobenpropit-induced IL-8 and TNF-α production was also investigated. P38 inhibitor SB203580 played an opposite way that significantly increased release of IL-8 and TNF-α from LAD2 cells induced by clobenpropit (Fig. 3B). What's more, after the treatment of an inhibitor of JNK (SP600125), the release of IL-8 and TNF-α induced via clobenpropit were not changed (Fig. 3C).

## Analysis of H4R induced activation of MAPKs, AKT, $Ca^{2+}$-Calcineurin-NFAT and NF-κB in human mast cells

The fact that some inhibitors could affect H4R induced IL-8 and TNF-α production indicated that these pathways may take part in the H4R signaling pathways. To figure out the signaling pathways used by H4R, we have analyzed the key molecules in activation of MAPKs, AKT, $Ca^{2+}$-Calcineurin-NFAT and NF-κB signaling pathways in LAD2 cells. LAD2 cells were pretreated with clobenpropit, and the changes in phospho-

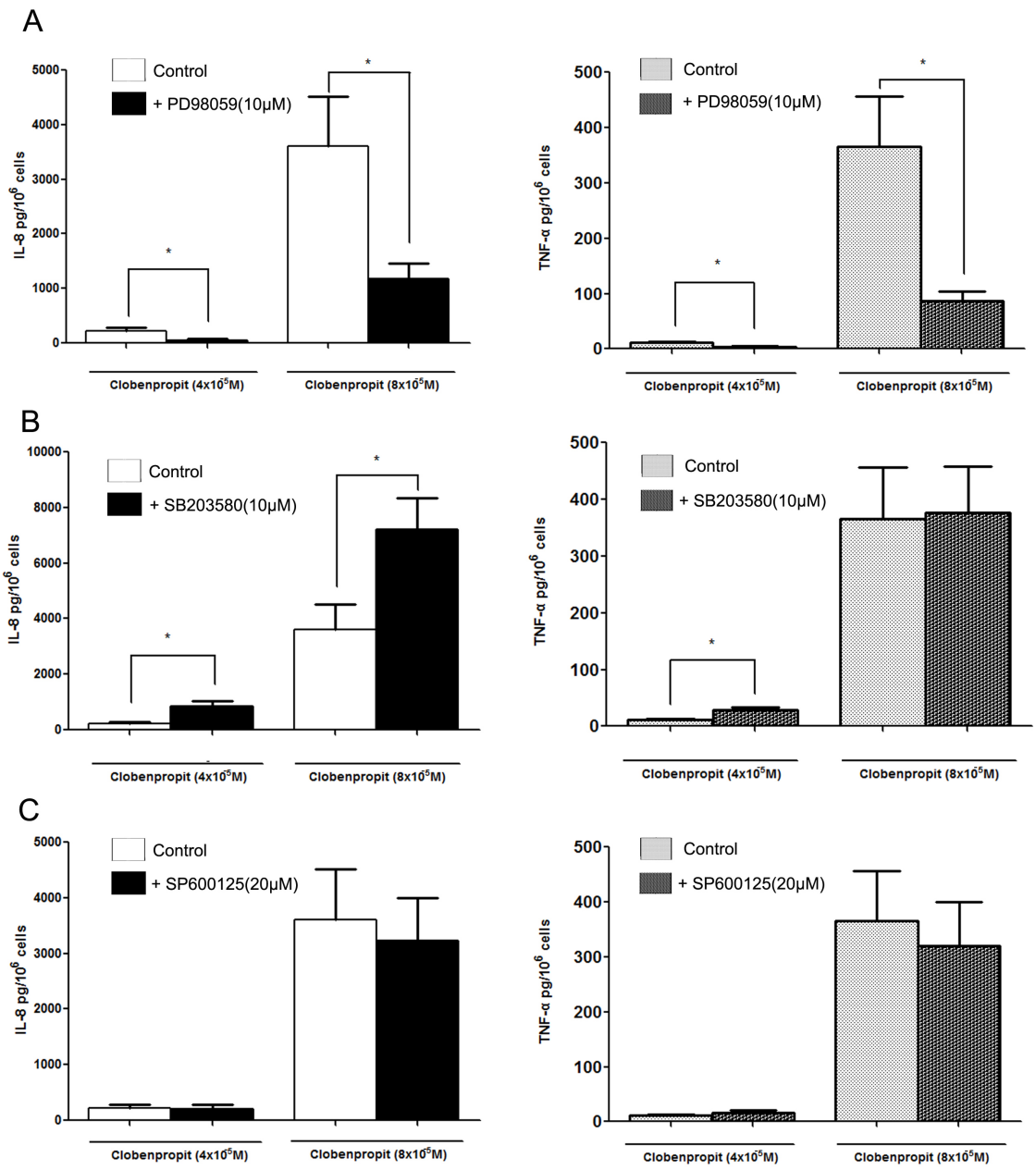


**Figure 3.** Effects of inhibitors of MAPKs on H4R agonist induced IL-8 and TNF-α release from LAD2 cells. (A) ERK inhibitor, PD98059 (10 μM), (B) p38 inhibitor, SB203580 (10 μM) or (C) JNK inhibitor, SP600125 (20 μM) was incubated with LAD2 cells for 30 min before the addition of two concentration of clobenpropit ($4\times10^{-5}$ M and $8\times10^{-5}$ M) for 24 h to induce the release of IL-8 and TNF-α. The amounts of IL-8 and TNF-α release from activated LAD2 cells pre-incubated with an inhibitor and the corresponding control pre-incubated in culture medium were compared with student t-test. *$p<0.05$ (n = 4–5).

rylation or location of the key proteins were observed.

As Fig. 4A shown, clobenpropit clearly induced ERK phosphorylation in mast cells. Phosphorylated AKT is a representative for activation of the PI3K pathway. AKT phosphorylation could be also induced in LAD2 cells activated by H4R agonist (Fig. 4A). What's more, H4R agonist was able to induce the phosphorylation of p38 in a time-dependent way in LAD2 cells.

Dephosphorylation of the transcription factor NFAT by the phosphatase calcineurin enables its nuclear translocation (22) . As shown in Fig. 4B, clobenpropit clearly increased nuclear translocation of NFAT in these mast cells. What's more, since there were no changes of IL-8 and TNF-α release from LAD2 cells incubated with the NF-κB inhibitor and JNK inhibitor, there were no obvious changes in the level of the NF-κB nuclear translocation and the JNK phosphorylation.

## Discussion

Histamine activates mast cells via H4R, which triggers many immune and inflammatory responses (2). However, the biological function of histamine H4 receptor was not clear. IL-8 and TNF-α were associated with allergic symptoms and chronic inflammatory conditions. Therefore, our data clearly showed that activation of the H4R led to IL-8 and TNF-α production. The effects of the H4R on IL-8 and TNF-α production were consistent with previous findings by using another H4R agonist, 4-methylhistamine (14).

Based on the previous clinical studies, H4R is a promising target in chronic inflammatory conditions (23). Recently, it indicated H4R played a role in a powerful inflammatory cascade, though the underlying mechanisms were unclear. The previous findings suggested that both ERK and PI3K phosphorylation were required

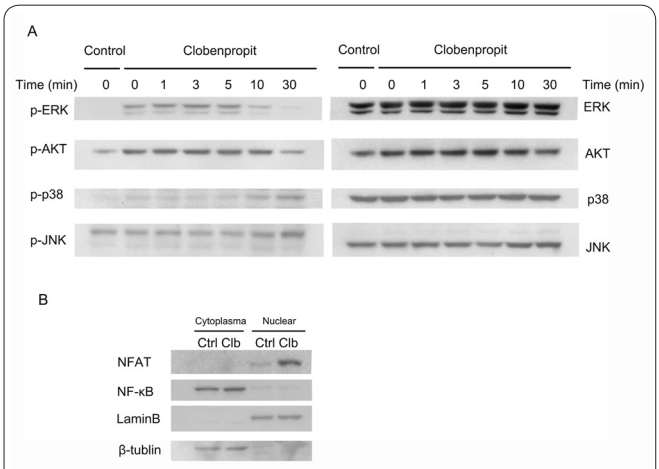


**Figure 4.** Time course of H4R agonist induced phosphorylation levels of kinase and subcellular location of transcription factor. (A) The phosphorylation levels of ERK, Akt, p38, JNK changed in the H4R agonist activation process. Mast cells were stimulated with H4R agonist clobenpropit at six different time intervals (0 min, 1 min, 3 min, 5 min, 10 min, 30 min). Blots carried out on total protein collected from LAD2 cells showed H4R induced the phosphorylation of Akt, ERK and P38. (B) Effect of H4R agonist on subcellular location of NF-κB and NFAT in human mast cells. Mast cells were pretreated with clobenpropit for 30 min. Blots carried out on cytoplasmic protein and nuclear protein collected from LAD2 cells showed H4R activates NFAT signaling pathway.

for the release of IL-6 via the activation of H4R on mast cells (9). The results shown here expanded on early reports of the H4R downstream signaling pathways (6, 10, 11, 24) .Our data suggested that activation of PI3K, ERK, $Ca^{2+}$-Calcineurin-NFAT and P38 signaling pathways were necessary for the IL-8 and TNF-α release induced via H4R in mast cells.

The kinetics of ERK, p38, JNK and AKT activation are also important in many cellular functions. There are several reports indicating the involvement of these kinase pathways in downstream of H4R. Pretreatment with pertussis toxin essentially abolished the ability to induce phosphorylation of ERK in the cells transfected with the H4R (24). Phosphorylated ERK also took part in MMP-9 production in endothelial cells, and enhanced survival of mature B cells (25, 26). It suggested that there were differential roles between transient activation and sustained activation of the PI3K pathway (27). Changes in kinetics of these kinases may influence the resident time of them and the location of their associated transcription factors, which lead to changes of IL-8 and TNF-α release.

However, there were some opposite evidences existing. For example, histamine could not induce ERK phosphorylation and instead histamine receptor could activate the transcription factor AP-1 in dendritic cells (10). What's more, p38 inhibitor seemed to partially reduce IL-6 level induced by histamine, which was not statistically significant (9). Because the inflammatory cascades in different inflammatory cells are complicated, the fine mechanism of cytokines and chemokines release in human mast cells via H4R requires further investigation.

## Acknowledges

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