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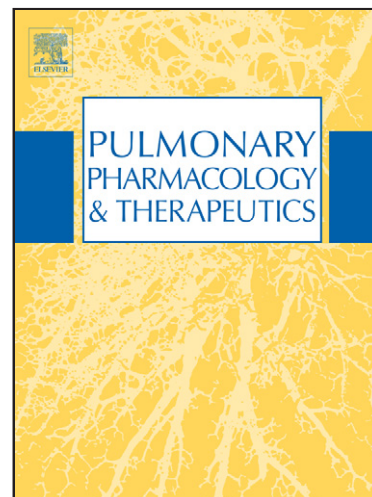
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**Histamine Reverses IL-5-Afforded Human Eosinophil Survival  
by Inducing Apoptosis: Pharmacological Evidence  
for a Novel Mechanism of Action of Histamine**

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**Abbreviations:** AC, adenylyl cyclase; cAMP, cyclic adenosine-3',5'-monophosphate; CI, confidence interval; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; DPPE, N,N-Diethyl-2-[4-(phenylmethyl)phenoxy]ethanamide; H<sub>1C</sub>-antagonist; H<sub>1C</sub>, intracellular histamine

“receptor”; HBSS, Hank’s balanced salt solution; 5-HT, 5-hydroxytryptamine; IL, interleukin; IP<sub>3</sub>, inositol(1,4,5)trisphosphate; JNJ7777120, 1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine, H<sub>4</sub>-antagonist; MMPCI, 7-methyl-2-[(4-methylpiperazin-1-yl)carbonyl]-1H-indole, H<sub>4</sub>-antagonist; OUP-16, (-)-2-Cyano-1-methyl-3-[(2R,5R)-5-[1H-imidazol-4(5-yl)]tetrahydrofuran-2-yl]methylguanidine, H<sub>4</sub>-agonist; PI, propidium iodide; PIP<sub>2</sub>, phosphatidylinositol(4,5)bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin.

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## Abstract

Eosinophils are essential inflammatory cells in the pathogenesis of asthma and atopic conditions. Histamine, released from mast cells and basophils in response to allergen exposure, is a critical mediator in the allergic response. Histamine exerts its effects via four unequivocally characterized histamine receptors, H<sub>1-4</sub>. Several functions of eosinophils have previously been shown to be stimulated by histamine. However, its effects on eosinophil apoptosis are unknown. The aim of the present study was to resolve the effects of histamine on constitutive apoptosis of human eosinophils and on the survival-enhancing action of interleukin (IL)-5. Additional experiments were conducted to elucidate the histamine receptor(s) involved in any response seen and the associated signal transduction cascade.

Human isolated peripheral blood eosinophils were cultured in the absence or presence of histamine, IL-5 and receptor antagonists/agonists or mediator inhibitors/analogues. Apoptosis was assessed by measuring the relative DNA content of propidium iodide (PI)-stained cells and the effects were confirmed by morphological analysis with bright field microscopy. Caspase activities were assessed by using commercial Caspase-Glo<sup>®</sup> 3/7, 8, and 9 luminescence assays.

Histamine (10-100  $\mu$ M) partially reversed IL-5-induced human eosinophil survival by enhancing apoptosis as assessed by measuring the relative DNA content of PI-stained cells. This effect was not mediated through any of the known histamine receptors or through non-specific activation of 5-hydroxytryptamine receptors or  $\alpha$ -adrenoceptors. Moreover, the reversal of IL-5-inhibited eosinophil apoptosis by histamine seemed not to utilize the conventional intracellular second messenger pathways including cyclic AMP, protein kinase A or phospholipase C. Inhibition of caspase 6 and caspases 1, 10 or 12 reversed the effects of histamine but also inhibited apoptosis in general.

In conclusion, the data presented herein indicate that histamine induces human eosinophil apoptosis in the presence of a survival-prolonging cytokine by a mechanism that does not apparently involve the activation of any of the currently known histamine receptor subtypes. The possibility exists that another, as yet unidentified, histamine receptor may exist in human eosinophils that regulates survival, although the participation of histamine receptor-independent mechanisms cannot be excluded.

**Key Words:** histamine, histamine receptors, eosinophils, apoptosis, IL-5, caspases

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## 1. Introduction

Eosinophilic inflammation represents an essential element in the pathogenesis of allergic diseases such as asthma and allergic rhinitis.[1-3] The balance between eosinophil maturation, recruitment and removal largely determines the number of eosinophils in the blood and tissues.[3,4] Apoptosis, or programmed cell death, is a controlled process of cell suicide that allows the removal of senescent cells or those whose continued survival would be detrimental for the organism. Furthermore, failure in the apoptotic process of eosinophils has been associated with pulmonary and allergic diseases.[3,5] Apoptosis is characterized by specific morphological and biochemical changes such as cell shrinkage, nuclear coalescence, chromatin condensation and endonuclease-catalyzed DNA breakdown followed by fragmentation of the cell into apoptotic bodies, which are phagocytosed intact without further induction of inflammation.[6,7] Apoptosis and its induction is regarded as an important mechanism in the resolution of eosinophilic inflammation [3,5,6] and it has been shown that eosinophil apoptosis is delayed in patients with asthma, inhalant allergy and atopic dermatitis [8,9]. *In vitro*, eosinophil apoptosis is inhibited by cytokines such as interleukin (IL) -3, IL-5 and granulocyte/macrophage colony-stimulating factor.[1,3]

Histamine is an important physiological amine that plays a major role in many physiological and pathological processes. Histamine was originally considered a mediator of acute inflammatory and immediate hypersensitivity reactions but is now believed to also affect chronic inflammatory processes and to regulate the immune response.[10,11] The effects of histamine are mediated through at least four distinct cell surface, G-protein-coupled receptors (histamine H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub> and the newly identified H<sub>4</sub>) [12-17] and eosinophils have been suggested to express all of these subtypes [10,11]. A fifth histamine receptor, located intracellularly and denoted histamine H<sub>1C</sub>, has also been described in haematopoietic cells.[18] Histamine binds to the different receptor subtypes with markedly different affinity: the K<sub>i</sub>-values range from 5-15 nM for the histamine H<sub>3</sub> and H<sub>4</sub> receptor subtypes [10,11,19] to approximately 10-100 μM for histamine H<sub>1</sub>, H<sub>2</sub> and H<sub>1C</sub> [10,11,18,19]. The four cell surface histamine receptor subtypes transduce extracellular signals by using a variety of G-proteins and intracellular second-messenger systems. Through the G<sub>q/11</sub>-coupled histamine H<sub>1</sub> receptor, histamine is thought to produce the symptoms and signs of allergy via stimulation of phospholipase C (PLC), which catalyzes the breakdown of phosphatidylinositol(4,5)bisphosphate (PIP<sub>2</sub>) to diacylglycerol (DAG) and

inositol(1,4,5)trisphosphate (IP<sub>3</sub>) that, in turn, activate protein kinase C (PKC) and mobilize Ca<sup>2+</sup> from intracellular stores, respectively.[10,11] The histamine H<sub>2</sub> receptor is positively coupled through G<sub>s</sub> to adenylyl cyclase (AC) and mediates the formation of cyclic adenosine-3',5'-monophosphate (cAMP). The histamine H<sub>2</sub> receptor is traditionally associated with mediating the enhancement of gastric acid secretion but has recently also been suggested to participate in the suppression of inflammation.[10,11] The histamine H<sub>3</sub> and H<sub>4</sub> receptors are both coupled to G<sub>i/o</sub> and inhibit AC activity, suppress the formation of cAMP and the activity of protein kinase A (PKA). The histamine H<sub>3</sub> receptor is mainly expressed in the nervous system and controls the release of histamine and other neurotransmitters whereas the histamine H<sub>4</sub> receptor has been suggested to be involved in immune regulatory functions such as chemotaxis and cytokine secretion.[10,11]

Histamine affects several essential functions of eosinophils. Allergen-induced accumulation of eosinophils in the airways, nose and skin can be inhibited by histamine H<sub>1</sub> receptor antagonists.[10,11] Histamine also affects eosinophil migration: histamine, at high concentrations, was previously suggested to inhibit eosinophil chemotaxis via the histamine H<sub>2</sub> receptor whereas at low concentrations, histamine was suggested to enhance chemotaxis via the histamine H<sub>1</sub> receptor.[10,11] However, it has recently been suggested that the newly identified histamine H<sub>4</sub> receptor is responsible for the selective recruitment and chemotaxis of eosinophils.[20] In addition, through the histamine H<sub>4</sub> receptor, histamine has also been found to mediate cell shape change, upregulation of adhesion molecules and to induce actin polymerisation and intracellular calcium mobilization in eosinophils.[21,22] Furthermore, histamine inhibits eosinophil degranulation via stimulation of the histamine H<sub>2</sub> receptors and a consequent elevation of intracellular levels of cAMP.[23]

The effects of histamine on granulocyte apoptosis remain scantily studied. Histamine, at high concentrations, has been suggested to induce human neutrophil apoptosis which could be reversed by histamine H<sub>1</sub> and H<sub>2</sub> receptor antagonists as well as with a pan-caspase inhibitor and an inhibitor of PKC.[24] There are no published data on the effect of histamine on human eosinophil apoptosis although it has been suggested that histamine does not affect eosinophil survival in the absence of cytokines.[25] It has, however, been described that the antihistamines (*aka* histamine H<sub>1</sub> receptor antagonists) oxatomide [26], fexofenadine [27] and diphenhydramine and chlorpheniramine [28] promote human eosinophil apoptosis whereas ketotifen [29] has been shown to induce primary necrosis in human eosinophils. The results

describing that histamine H<sub>1</sub> receptor antagonists influence human eosinophil survival suggest that histamine itself may also have a role in the regulation of human eosinophil apoptosis. Thus, studies on the effects of histamine on human eosinophils are warranted.

The objective of the present study described herein was to resolve whether histamine affects constitutive apoptosis or IL-5-induced survival of human eosinophils and, if so, to elucidate the receptor(s) and signalling pathway involved.

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## 2. Materials and Methods

### 2.1 Eosinophil Isolation and Culture

One hundred millilitres of peripheral venous blood was obtained from volunteers with normal or slightly elevated blood eosinophil counts. The volunteers were healthy or atopic. Subjects with hypereosinophilic syndrome were excluded. Before donating blood, the subjects gave written informed consent to the study protocol approved by the Ethics committee of Tampere University Hospital (Tampere, Finland). Eosinophils were isolated under sterile conditions to >99% purity by using CD16-negative immunomagnetic bead selection as previously described.[8,30-32] Isolated eosinophils were cultured for 40 h (+37°C, 5% CO<sub>2</sub>) in the absence or presence of histamine, IL-5 and other selected compounds as indicated in RPMI 1640 medium (Dutch modification) with 10% fetal bovine serum and antibiotics unless otherwise stated.

### 2.2 Determination of Apoptosis by the Relative DNA Content Assay by Flow Cytometry

The number of apoptotic cells was assessed by measuring the relative DNA content by flow cytometry (FACScan, Becton Dickinson, San Jose, CA) of propidium iodide (PI)-stained cells.[8,30-32] Endonuclease-catalysed DNA fragmentation is regarded as a specific feature of apoptosis.[33] Therefore, the cells showing decreased relative DNA content were considered to be apoptotic. Eosinophils were suspended in 300 µl of hypotonic PI solution (25 µg ml<sup>-1</sup> in 0.1% sodium citrate and 0.1% Triton X-100), protected from light and incubated at +4°C for 1 h before flow cytometric analysis.

### 2.3 Morphological Analysis

To confirm the mode of cell death, eosinophil morphology was assessed by bright field microscopy. For morphological analysis, eosinophils were spun onto cytospin slides (500 rev min<sup>-1</sup>, 5 min) and stained with May-Grünwald-Giemsa after fixation in methanol. Cells showing the characteristic apoptotic morphology, such as cell shrinkage, nuclear coalescence and chromatin condensation, were considered to have undergone apoptosis.

## 2.4 Caspase Activity Assay

Caspase 3/7, 8 and 9 activities in eosinophils were assessed by using Caspase-Glo<sup>®</sup> 3/7, 8, and 9 assays (Promega Corp., Madison, USA) according to the manufacturer's instructions. Briefly, human eosinophils were cultured in RPMI 1640 medium (Dutch modification) with 10% fetal bovine serum and antibiotics in the absence and presence of IL-5 (1 pM) and histamine (100  $\mu$ M) for 16 h. Equal volumes of Caspase-Glo<sup>®</sup> 3/7, 8 or 9 reagents were added after which the samples were incubated for 1 h at room temperature before measurement of luminescence.

## 2.5 Materials

Histamine, mepyramine (pyrilamine maleate), cimetidine, thioperamide maleate, Rp-8-CPT-cAMPS, 8-Br-cAMP, methysergide maleate, 5-hydroxytryptamine (5-HT) hydrochloride, phentolamine hydrochloride, ( $\pm$ )-adrenaline hydrochloride, PI, JNJ7777120 (1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine), dexamethasone and pertussis toxin (PTX) and were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Z-D(OMe)QMD(OMe)-FMK, Z-VEID-FMK, Ac-IETD-CHO, Ac-LEHD-CHO and Q-VD-OPh were obtained from Merck (Darmstadt, Germany). Other reagents were obtained from the following sources: Z-Asp-CH<sub>2</sub>-DCB (Peptide Institute, Inc., Osaka, Japan), Caspase-Glo<sup>®</sup> 3/7, 8, 9 assays (Promega Corp., Madison, WI, USA), cirazoline hydrochloride, clonidine hydrochloride, D609, U73122, DPPE fumarate (N,N-Diethyl-2-[4-(phenylmethyl)phenoxy]ethanamide fumarate) and clobenpropit dihydrobromide (Tocris Cookson Ltd., Avonmouth, UK), anti-CD16 microbeads and the magnetic cell separation system (Miltenyi Biotec, Bergish Gladbach, Germany), human recombinant IL-5 (R&D system Europe, Abingdon, UK), Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), antibiotics and RPMI 1640 (Dutch modification) (Gibco BRL, Paisley, UK), fetal bovine serum (Euroclone, Pero, Italy), Hank's balanced salt solution (HBSS) and RPMI 1640 (BioWhittaker, Verviers, Belgium), May-Grünwald (Merck, Darmstadt, Germany) and Giemsa (J.T. Baker, Deventer, Holland). (-)-2-Cyano-1-methyl-3-{{(2R,5R)-5-[1H-imidazol-4(5)-yl]tetrahydrofuran-2-yl}methylguanidine (OUP-16) was a kind gift from Prof. Atsushi Yamatodani and Shinya Harusawa from the University of Osaka, Japan. H<sub>4</sub>-antagonist 7-methyl-2-[(4-methylpiperazin-1-yl)carbonyl]-1H-indole (MMPCI) was a kind gift from Dr. Michael Peck from UCB Pharma (Brussels, Belgium).

Histamine, mepyramine, cimetidine, thioperamide, 5-HT, D609, adrenaline, cirazoline and clonidine were dissolved in RPMI 1640 medium (Dutch modification). Clobenpropit, DPPE, Rp-8-CPT-cAMPS, 8-Br-cAMP and dexamethasone were dissolved in HBSS. OUP-16, MMPCI, JNJ7777120, U73122, methysergide and all caspase inhibitors were dissolved in dimethyl sulfoxide (DMSO). The final concentration on DMSO in culture was 0.5% except for Z-Asp-CH<sub>2</sub>-DCB for which the final concentration of DMSO within the cells was 1.0%. 0.5-1% DMSO was not found to affect eosinophil viability as assessed by bright field microscopy (n=6, data not shown). Similar concentrations of DMSO were added to the control cultures. Stock solution of phentolamine was prepared in ethanol. The final concentration of ethanol in the culture was 0.1%, and a similar concentration of ethanol was added to the control cultures. 0.1% ethanol did not affect eosinophil viability as assessed by flow cytometry (n=4, data not shown). Pertussis toxin was dissolved in 50% (v/v) glycerol containing 50 mM Tris, pH 7.5, 10 mM glycine and 0.5 M NaCl. Similar solution was also added to the control, and was not found to affect eosinophil viability as assessed by flow cytometry (n=5, data not shown).

## 2.6 Statistics

The results are expressed as the mean  $\pm$  SEM. Apoptosis is expressed as apoptotic index (number of apoptotic cells/total number of cells, i.e. apoptotic index 0.1 means that 10% of the cells are apoptotic). Statistical significance was calculated by analysis of variance for repeated measures supported by the Dunnett test or by paired t-tests by using GraphPad Instat software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when  $p < 0.05$ . The concentration-response data of histamine-induced apoptosis, the EC<sub>50</sub> (effective concentration that produces 50% of the maximal effect) value and the 95% confidence interval (CI) were analyzed by using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

### 3. Results

#### 3.1 Effect of Histamine on Human Eosinophil Apoptosis

##### 3.1.1 Constitutive Apoptosis

Human eosinophils cultured for 40 h in cytokine-deprived conditions underwent spontaneous apoptosis (apoptotic index  $0.66 \pm 0.07$ ) as defined by relative DNA fragmentation. When different concentrations (0.01-100  $\mu\text{M}$ ) of histamine were added to culture, no alterations in the rate of apoptosis were seen ( $n=6$ , Fig. 1A). Histamine (100  $\mu\text{M}$ ) consistently had no effect on eosinophil apoptosis in the absence of cytokines when apoptosis was assessed by bright field microscopy (apoptotic indices were  $0.50 \pm 0.07$  and  $0.41 \pm 0.06$  with and without histamine, respectively,  $n=6$ ,  $p>0.05$ ).

##### 3.1.2 IL-5-Induced Eosinophil Survival

Culture of human eosinophils for 40 h in the presence of 1 pM IL-5 markedly enhanced eosinophil survival and attenuated the apoptotic index to  $0.15 \pm 0.02$ . Histamine (0.01 to 100  $\mu\text{M}$ ) partially reversed IL-5-induced eosinophil survival by enhancing apoptosis ( $n=6$ , Fig. 1B, E and F). The enhancement of human eosinophil apoptosis by histamine was concentration-dependent ( $\text{EC}_{50} = 0.56 \mu\text{M}$  [CI = 0.33-0.97  $\mu\text{M}$ ], Fig. 1C insert) and reached a magnitude of approximately 40% at concentrations of histamine between 10-100  $\mu\text{M}$  (Fig. 1C). The magnitude of the apoptosis-promoting effect of histamine in the presence of IL-5 was similar to the effect of dexamethasone ( $n=4$ , Fig. 1D). The mode of cell death was confirmed by bright field microscopy and 100  $\mu\text{M}$  histamine was found to increase eosinophil apoptosis by 56% in the presence of 1 pM IL-5. The apoptotic indices were  $0.08 \pm 0.02$  and  $0.13 \pm 0.07$  in the absence and presence of histamine (100  $\mu\text{M}$ ), respectively ( $n=6$ ,  $p<0.01$ ).

Similarly to the apoptosis-enhancing effect of glucocorticoids, which is abolished by higher concentrations of cytokines [3], the effect of histamine in reversing IL-5-mediated eosinophil survival also fell off when the concentration of IL-5 was increased ten-fold. In the presence of 10 pM IL-5, 10  $\mu\text{M}$  histamine increased eosinophil apoptosis by approximately 40% but this effect was not quite statistically significant due to higher variability ( $n=6$ ,  $p>0.05$ , data not

shown). Thus, subsequent experiments were performed by using the lower (1 pM) concentration of IL-5.

### *3.2 Role of Caspases in Histamine-Induced Human Eosinophil Apoptosis*

#### *3.2.1 Caspase Activities*

To assess the role of caspases in histamine-induced reversal of IL-5-afforded eosinophil survival, the activities of caspases 3/7, 8 and 9 were measured by using Caspase-Glo<sup>®</sup> 3/7, 8, and 9 assays. Active caspases 3/7, 8 and 9 were detected after 16 h incubation. Activities of all caspases 3/7, 8 and 9 were markedly reduced by 1 pM IL-5 (Fig. 2). Histamine (100  $\mu$ M) was found to slightly increase the activities of caspases 3/7 and 9 in the presence of 1 pM IL-5 (Fig. 2).

#### *3.2.2 Effect of Caspase-Inhibitors on Histamine-Induced Apoptosis*

To further evaluate the role of different caspases in histamine (100  $\mu$ M)-induced eosinophil apoptosis, the effects of various caspase-inhibitors in the absence and presence of IL-5 (1 pM) were evaluated. Unexpectedly, a pan-caspase inhibitor, Z-Asp-CH<sub>2</sub>-DCB (200  $\mu$ M), further enhanced histamine-induced apoptosis in the presence of IL-5 (Fig. 3) whereas a broad-range inhibitor of caspases 1, 3, 8, 9, 10 and 12, Q-VD-Oph (20  $\mu$ M), reversed histamine-induced apoptosis in the presence of IL-5. The mechanism of this phenomenon remains obscure. An analogous effect to Q-VD-Oph was found with the caspase 6 inhibitor Z-VEID-FMK (200  $\mu$ M) (Fig. 3), i.e. reversal of histamine-induced apoptosis. In contrast, inhibitors of caspases 3 (Z-DQMD-FMK, 200  $\mu$ M), 8 (Ac-IETD-CHO, 100  $\mu$ M) and 9 (Ac-LEHD-CHO, 100  $\mu$ M) did not exert any effects on eosinophil apoptosis (Fig. 3). In addition, Q-VD-Oph and Z-VEID-FMK reduced apoptosis in histamine-treated cells in the absence of IL-5 (Fig. 3).

### 3.3 Role of Histamine Receptors in Histamine-Induced Human Eosinophil Apoptosis

#### 3.3.1 Histamine H<sub>1</sub>, H<sub>2</sub> and H<sub>3</sub> Receptor Subtypes

Pre-treatment of eosinophils for 15 min with mepyramine (1  $\mu$ M, a histamine H<sub>1</sub> receptor antagonist - K<sub>i</sub>  $\sim$  1 nM [34]), cimetidine (10  $\mu$ M, a histamine H<sub>2</sub> receptor antagonist - K<sub>i</sub>  $\sim$  0.8  $\mu$ M [34]) and thioperamide (10 nM, a histamine H<sub>3</sub> receptor antagonist - K<sub>i</sub>  $\sim$  10 nM [19]) or different combinations of these antagonists had no effect on the survival-enhancing activity of IL-5 (1 pM) in the absence or presence of histamine (100  $\mu$ M) (Fig. 4). Similarly, when the antagonists were used alone or in combinations, no significant effects on constitutive apoptosis or apoptosis in the presence of only histamine were observed ( $p > 0.05$ , data not shown) except for the combination of thioperamide and cimetidine, which slightly inhibited spontaneous eosinophil apoptosis ( $p < 0.05$ , data not shown).

#### 3.3.2 The Histamine H<sub>4</sub> Receptor Subtype

Thioperamide is also an antagonist of the histamine H<sub>4</sub> receptor at concentrations in the low micromolar range (K<sub>i</sub>  $\sim$  0.13  $\mu$ M) [13,16,17,19] and was examined on eosinophil apoptosis in the absence and presence of histamine (100  $\mu$ M) and IL-5 (1 pM). As shown in figure 5A, thioperamide did not reverse the effect of histamine but slightly attenuated the rate of apoptosis in general at the highest concentration (10  $\mu$ M) studied (Fig. 5A).

To further investigate the role of the H<sub>4</sub>-subtype, the effect of JNJ777120, a recently described selective histamine H<sub>4</sub> receptor antagonist (K<sub>i</sub>  $\sim$  4 nM) [35] was studied on human eosinophil apoptosis in the absence and presence of histamine and IL-5. At concentrations of JNJ777120 up to 500 nM, apoptosis of human eosinophils was unaffected (Fig. 5B). To exclude the possibility that the high concentration of histamine (100  $\mu$ M) used in these experiments would, by competition, limit the degree of antagonism produced by JNJ777120, we also tested the effect of 10  $\mu$ M JNJ777120 on eosinophil apoptosis induced by 10  $\mu$ M histamine in the presence of 1 pM IL-5. Again, under this experimental condition, the effect of histamine was not reversed ( $p > 0.05$ , data not shown). Similar results were obtained with another selective histamine H<sub>4</sub> receptor antagonist, MMPCI (pA<sub>2</sub> = 7.5), which is selective for the histamine H<sub>4</sub> receptor up to concentrations of 10  $\mu$ M (Michael Peck, UCB Pharma, personal communication). Thus, MMPCI did not reverse the apoptosis-promoting effect of

histamine on IL-5-afforded eosinophil survival (Fig. 5C). To eliminate the possibility that histamine at 100  $\mu\text{M}$  competed out the antagonist, the effect of MMPCI (10  $\mu\text{M}$ ) on 10  $\mu\text{M}$  histamine-induced eosinophil apoptosis in the presence of 1 pM IL-5 was studied and found not to reverse the pro-apoptotic effect of histamine ( $p>0.05$ , data not shown).

To confirm that the histamine  $H_4$  receptor was not responsible for the apoptosis-promoting effect of histamine on IL-5-induced survival of human eosinophils, two other sets of experiments were conducted. Thus, OUP-16, a histamine  $H_4$  receptor agonist [36] and clobenpropit, an  $H_3$  receptor antagonist with partial agonist action at the  $H_4$  receptor at concentrations above 1 nM [12,13,16,21], were examined to establish if they could promote apoptosis in a manner similar to histamine. However, neither OUP-16 (0.01-10  $\mu\text{M}$ ) nor clobenpropit (0.1-30 nM) affected apoptosis in the absence or presence of IL-5 ( $n=5-7$ ,  $p>0.05$ , data not shown) at concentrations that spanned their estimated  $K_{i5}$  (OUP-16 = 125 nM [37]; clobenpropit = 8 nM [19]).

### 3.3.3 Effect of a Histamine $H_{1C}$ "Receptor" Antagonist on Human Eosinophil Apoptosis

Histamine has also been described to have an intracellular binding site that has been denoted the histamine  $H_{1C}$  receptor, which is activated by micromolar concentrations of histamine.[18] To determine if histamine exerts its apoptosis-promoting effects via the intracellular histamine receptor, we tested the effect of DPPE, a putative antagonist of the histamine  $H_{1C}$  receptors [18], on eosinophil apoptosis in the absence and presence of histamine (100  $\mu\text{M}$ ) and IL-5 (1 pM). DPPE did not reverse the histamine-induced apoptosis ( $n=6$ ,  $p>0.05$ , data not shown) although spontaneous eosinophil apoptosis was slightly decreased as well as apoptosis seen in the presence of 100  $\mu\text{M}$  histamine without IL-5 ( $n=6$ ,  $p<0.01$ , data not shown).

### 3.4 Role of Different Signal Transduction Pathways in Histamine-Induced Human Eosinophil Apoptosis

Although none of the currently defined histamine receptor subtypes apparently mediated the ability of histamine to promote apoptosis of eosinophils cultured in the presence of IL-5, additional studies were performed to gain information that would support a receptor-mediated mechanism.

### 3.4.1 Effect of Pertussis Toxin on Histamine-Induced Apoptosis

Pretreatment of eosinophils with PTX, which inactivates  $G_{i/o}$ , at concentrations 0.1, 0.5 and  $1.0 \mu\text{g ml}^{-1}$  for 90 min at  $+37^\circ\text{C}$ , did not influence human eosinophil apoptosis under any conditions examined (Fig. 6A).

### 3.4.2 Role of cAMP and Protein Kinase A -Pathway in Histamine-Induced Apoptosis

The cell permeant cAMP analogue, 8-Br-cAMP (1 mM), significantly and markedly decreased constitutive eosinophil apoptosis ( $n=4$ ,  $p<0.001$ , data not shown) but had no effect on cell survival in the presence of IL-5 ( $n=4$ ,  $p>0.05$ , data not shown). Therefore, the effect of histamine on IL-5-mediated eosinophil survival was not mimicked by a cAMP analogue, indicating it was not due to an increase in the levels of cAMP in the cell. In addition, the PKA inhibitor, Rp-8-CPT-cAMPS, had no effect on eosinophil apoptosis in the absence or presence of histamine ( $100 \mu\text{M}$ ) and IL-5 (1 pM; Fig. 6B).

### 3.4.3 Role of Phospholipase C in Histamine-Induced Apoptosis

To evaluate the possible role of PLC in human eosinophil apoptosis, we tested two different pharmacological inhibitors, D609 and U73122. D609 had no effects on human eosinophil apoptosis whereas U73122 inhibited constitutive eosinophil apoptosis and decreased apoptosis in the presence of histamine without IL-5. However, in IL-5-treated cells, U73122 did not reverse the apoptosis-enhancing effect of histamine (Fig. 6C).

### 3.5 Role of 5-HT-Receptors and $\alpha$ -Adrenoceptors in Histamine-Induced Human Eosinophil Apoptosis

As relatively high concentrations of histamine were required to promote eosinophil apoptosis, we investigated whether other amine receptors, namely those activated by 5-HT and catecholamines could be involved in the action of histamine. Thus, we first examined the effect of methysergide, a nonselective 5-HT<sub>1</sub>-, 5-HT<sub>2</sub>- and 5-HT<sub>7</sub>-receptor antagonist as well as the natural ligand for these receptors, 5-HT, on eosinophil apoptosis. Methysergide ( $1-10 \mu\text{M}$ ) did not alter the rate of histamine-induced eosinophil apoptosis either in the absence or

presence of IL-5 (n=5, p>0.05, data not shown). Furthermore, 5-HT (1-100  $\mu$ M) did not mimic the apoptosis-promoting effect of histamine on IL-5-afforded eosinophil survival (n=4, p>0.05, data not shown).

Phentolamine (10  $\mu$ M), a non-selective  $\alpha$ -adrenoceptor antagonist, slightly decreased constitutive eosinophil apoptosis in the absence and presence of histamine (n=4, p<0.05, data not shown). However, no reversal of the enhancement of apoptosis by histamine in the presence of IL-5 was observed (n=4, p>0.05, data not shown). To further exclude the involvement of  $\alpha$ -adrenoceptors in mediating histamine-induced human eosinophil apoptosis, we tested the effects of adrenaline (0.1-1  $\mu$ M, a non-selective agonist), cirazoline (1-10  $\mu$ M, a selective  $\alpha_1$ -agonist) and clonidine (10-100  $\mu$ M, an  $\alpha_2$ -agonist) on apoptosis of human eosinophils in the absence and presence of IL-5. None of these pharmacological interventions increased apoptosis (n=4, p>0.05, data not shown) although a slight decrease in eosinophil survival in the absence of IL-5 was observed at high (100  $\mu$ M) concentrations of clonidine (n=4, p<0.05, data not shown).

#### 4. Discussion

In the present study we have shown that histamine partly reverses IL-5-afforded survival of human eosinophils by inducing apoptosis. This effect of histamine is not apparently mediated through any of the known histamine receptors or through agonism at 5-HT-receptors or  $\alpha$ -adrenoceptors. Moreover, the reversal of IL-5-inhibited eosinophil apoptosis by histamine does not utilize the common intracellular second messenger pathways including cAMP, PKA or PLC but may involve caspase 6 and at least some of caspases 1, 10 or 12. Thus, the results raise the possibility that human eosinophils may express a novel, hitherto unidentified, histamine receptor.

The increase in eosinophil apoptosis elicited by histamine in the presence of IL-5 reached a magnitude of 40%. This degree of reversibility is comparable to that produced by glucocorticoids, which is considered one of their key anti-inflammatory mechanisms in the treatment of eosinophilic conditions such as asthma.[3,38] However, histamine did not increase constitutive eosinophil apoptosis (unlike glucocorticoids), which implies that the mechanism of action differs between these two classes of compounds. The concentration of IL-5 (1 pM  $\approx$  30 pg ml<sup>-1</sup>) used in the present study and reversed by histamine is quite low although very potent in increasing eosinophil survival. Recent reports indicate that the plasma concentration of IL-5 is approximately 4 pg ml<sup>-1</sup> in healthy individuals [39] whereas the sputum concentrations of IL-5 are approximately 30 pg ml<sup>-1</sup> and 90 pg ml<sup>-1</sup> in healthy and asthmatic persons, respectively [40]. Thus, the used concentration of IL-5 is clinically relevant.

Previous studies have reported that histamine concentrations ranging from 10 nM to 10  $\mu$ M are required to achieve the histamine H<sub>4</sub> receptor-mediated effects on eosinophil chemotaxis, cell shape change, upregulation of adhesion molecules and intracellular calcium mobilization.[20-22] Furthermore, even higher concentrations of histamine have been reported to be necessary for the enhancement of apoptosis of neutrophils.[24] In addition, the histamine concentrations used in nasal [41,42] and inhalation [43] challenges are manifold as compared to the effective concentration used in this study. Therefore, it seems likely that levels of histamine similar to those used to induce apoptosis of cytokine-treated human eosinophils *in vitro* can be achieved locally after histamine challenges used in the diagnosis of asthma and allergic rhinitis, which may be relevant also *in vivo*. Moreover, during

inflammation, the local concentration of histamine released from basophils and mast cells has been estimated to be in the millimolar range acting on neighbouring cells such as eosinophils in an autocrine and paracrine fashion.[44] Taken together, this potentially anti-inflammatory action of histamine on cytokine-afforded eosinophil survival occurs at clinically relevant concentrations and the magnitude of this effect is equivalent to that evoked by glucocorticoids. Thus, we suggest that the reversal of IL-5-induced eosinophil survival by histamine represents a novel means of regulating the immune system. The response to histamine seen *in vivo* will derive from the net effect of those receptor subtypes that are activated, of which some effects are pro-inflammatory and others may be anti-inflammatory. It is possible that all histamine receptor subtypes are activated on a variety of cells during the allergic reaction and that blockage of the possible novel histamine receptor on eosinophils may enhance inflammation further.

Our data suggest that the ability of histamine to partially reverse IL-5-afforded inhibition of human eosinophil apoptosis is not mediated through any of the currently known histamine receptors. Given the diverse role of histamine through the newly identified histamine H<sub>4</sub> receptor in the regulation of inflammation [37], we hypothesized that the effect of histamine could be histamine H<sub>4</sub> receptor -mediated. However, experiments with selective agonists and antagonists failed to provide evidence that this effect of histamine was mediated through the histamine H<sub>4</sub> receptor subtype under the experimental conditions used. Similar studies with selective receptor antagonists also excluded a role of the histamine H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub> and H<sub>1C</sub> receptors. The histamine H<sub>1</sub> and H<sub>2</sub> receptor antagonists, mepyramine and cimetidine, were used at concentrations well in excess of their respective affinities at the H<sub>1</sub> and H<sub>2</sub> receptors [34] but lower than the concentrations likely to promote eosinophil apoptosis due to mechanisms unrelated to histamine receptor antagonism [26-28]. If either the H<sub>1</sub> or the H<sub>2</sub> receptor subtype was involved in the reversal of IL-5-mediated eosinophil survival, mepyramine or cimetidine would have been expected to antagonize the effect of histamine, which was used at a concentration approximately similar to its K<sub>i</sub> at the H<sub>1</sub> and H<sub>2</sub> receptors [19]. Were the histamine H<sub>4</sub> receptor subtype involved, significant agonism would have been predicted with clobenpropit and OUP-16 based on their affinities at the histamine H<sub>4</sub> receptor.[19,37] Moreover, at least some antagonism by thioperamide, JNJ7777120 and MMPCI would have been expected at the H<sub>4</sub> receptor [19,35] even though histamine was used with a much higher concentration than its affinity for the H<sub>4</sub> receptor subtype [19]. In previous studies evaluating the H<sub>4</sub> receptor-mediated effects of histamine on eosinophils, the

effects of similar histamine concentrations were antagonized with similar thioperamide and JNJ7777120 concentrations and agonized with similar clobenpropit concentrations as used in the present study.[21,22] Thus, the present results indicate that the histamine H<sub>4</sub> receptor subtype does not mediate the apoptosis-enhancing effect of histamine on IL-5-induced human eosinophil survival.

Regarding the histamine H<sub>3</sub> receptor subtype, significant antagonism of histamine-induced apoptosis may not have occurred with thioperamide due to competition.[19] However, evidence suggesting the existence of the histamine H<sub>3</sub> receptors on human eosinophils is now known to be incorrect.[1,45,46] Indeed, the novel histamine receptor discovered in eosinophils by Raible and co-workers in the 1990s [45,46] has now been identified as the H<sub>4</sub> subtype [12,13,16,17,20,21,37,47]. Moreover, human eosinophils have recently been shown not to express the histamine H<sub>3</sub> receptor.[48] Thus, histamine-induced human eosinophil apoptosis in the presence of IL-5 is unlikely to be mediated through the histamine H<sub>3</sub> receptor subtype.

As relatively high concentrations of histamine were required to promote apoptosis of IL-5-treated eosinophils, the possibility that histamine may act through other amine receptors was evaluated. In particular, given the possible role of 5-HT in asthma [49,50] and because 5-HT has been suggested to act as an eosinophil chemoattractant via the 5-HT<sub>2A</sub>-receptor [51], we explored whether 5-HT-receptors could mediate the pro-apoptotic effect of histamine on eosinophils. However, the 5-HT-receptor antagonist, methysergide, did not block histamine-induced eosinophil apoptosis nor did 5-HT mimic the effects of histamine. The role of  $\alpha$ -adrenoceptors was also evaluated. Neither selective (cirazoline, clonidine) nor non-selective (adrenaline)  $\alpha$ -adrenoceptor agonists emulated the effect of histamine. Phentolamine, an  $\alpha$ -adrenergic antagonist, did not reverse the apoptosis-enhancing effect of histamine on IL-5-mediated human eosinophil survival either. Additional studies, performed to determine if common intracellular second messenger signalling pathways could account for the ability of histamine to promote apoptosis, excluded a role for PTX-sensitive pathways, cAMP, PKA and PLC.

It has been proposed that additional histamine receptors remain to be discovered as not all effects of histamine can yet be explained by the currently defined subtypes.[47] Our result demonstrating the partial reversal of cytokine-afforded survival of human eosinophils by

histamine through induction of apoptosis represents yet another effect of histamine that can not be explained by any of the currently known histamine receptors or by activation of other amine receptors on eosinophils. These data further suggest the possible existence of an additional receptor for histamine although a histamine receptor-independent mechanism cannot be excluded. Multiple other contingencies also exist that may mediate histamine-induced enhancement of human eosinophil apoptosis in the presence of IL-5. For instance, histamine may, in some unknown way, modify the signalling cascades activated by IL-5 in human eosinophils. Although the detailed signalling pathways involved in cytokine-induced eosinophil survival are not fully understood, cytokine receptors form dimers and phosphorylate tyrosine residues upon activation. The subsequent signalling events include adaptor proteins, Ras – mitogen-activated protein kinase pathways and Janus kinase – signal transducer and activator of transcription pathways.[3] Histamine might also stimulate the production of another mediator or cytokine that interferes with IL-5-mediated eosinophil survival. For example, prostaglandin D<sub>2</sub> [52] and IL-4 [53] could be such mediators as they have been reported to induce eosinophil apoptosis. Histamine may also promote eosinophil apoptosis through a non-histamine-receptor that is not a 5-HT or alpha variant nor is a classical 7-span G-protein-coupled receptor. For example, an ion-channel receptor or a nuclear receptor might be mediating the histamine-effect. Alternatively, histamine may utilize non-receptor-mediated mechanisms. Thus, detailed additional experiments focusing on the mechanism of histamine-induced reversal of IL-5-afforded inhibition of human eosinophil apoptosis are highly warranted.

Regulation of caspase activity is considered essential in the apoptotic process. Caspases are constitutively expressed cysteine proteases that cleave substrates at specific aspartic acid residues. In the resting cell, caspases are normally present as inactive proenzymes that are activated after cleavage at internal aspartate residues to form the mature protein.[54] During apoptosis, caspases are activated in an amplifying cascade and can be divided into two functionally different subgroups, the initiator and effector caspases.[54] At present, the specific caspase pathways mediating eosinophil apoptosis remain poorly characterized.[3] However, the presence of caspases 3, 6, 7, 8 and 9 has been described in eosinophils and spontaneous eosinophil apoptosis seems to utilize these caspases.[3] Recently, caspases 3 and 6 have been suggested to play a role in orazipone-induced human eosinophil apoptosis.[55] The present results suggest that caspase 6 and at least some of caspases 1, 10 or 12 may mediate the apoptosis-promoting effect of histamine on IL-5-treated human eosinophils. In

contrast, caspases 3 and 9 seem to be unimportant in histamine-induced reversal of cytokine-afforded eosinophil survival despite their activation during apoptosis.

In interpreting the current results, the existence of different subpopulations of eosinophils is a possibility also worth mentioning. For instance, a small proportion of eosinophils have been shown to express CD16 upon activation in allergic patients [56]. Thus, during eosinophil isolation with the CD16-negative selection method, we may have lost this part of the eosinophil population. Whether these cells have different responses to histamine or possess a different histamine receptor expression profile than those in the present study remains to be elucidated.

In conclusion, our results demonstrate that histamine partially reverses IL-5-afforded survival of human eosinophils by inducing apoptosis and the magnitude of this effect mirrors that produced by glucocorticoids. The pro-apoptotic action of histamine occurs at clinically relevant concentrations and represents a novel mechanism of regulation within the immune system. The effect of histamine on eosinophil apoptosis cannot be explained by any of the currently known histamine receptors or by non-specific activation of other amine receptors on eosinophils. Thus, although we cannot exclude the possibility that histamine-induced apoptosis occurs through a receptor-independent mechanism, the data presented herein implicate the participation of a novel histamine receptor subtype.

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## Figure Captions

**Figure 1. Effect of histamine on human eosinophil apoptosis.** The apoptotic indices of eosinophils cultured with histamine in the absence (A) and presence (B) of 1 pM IL-5 are shown. Percentage changes in apoptosis by different histamine concentrations in the presence (C) of 1 pM IL-5. In (C), the insert shows the concentration-response curve of histamine-induced apoptosis. In (D), the effect of dexamethasone on constitutive and IL-5-inhibited eosinophil apoptosis is shown for comparison. The flow cytometry histograms of one experiment representative of six essentially identical experiments of 1 pM IL-5-induced eosinophil survival (E) and 100  $\mu$ M histamine-induced partial reversal of 1 pM IL-5-mediated eosinophil survival (F) are also shown. The figure in the top right corner (E-F) represents the percentage of apoptotic cells. Apoptosis was analyzed by the relative DNA fragmentation assay. Each data point (A-D) represents the mean  $\pm$  SEM of n=4-6 independent determinations using eosinophils from different donors. \*\* indicates p<0.01 as compared with the respective control.

**Figure 2. Effect of histamine (100  $\mu$ M) on caspase 3/7, 8 and 9 activation in human eosinophils in the presence of IL-5 (1 pM) after a 16 h incubation.** Caspase activities were measured with Caspase-Glo<sup>®</sup> 3/7, 8, and 9 assays. Each data point represents the mean  $\pm$  SEM of n=6 independent determinations using eosinophils from different donors. \* indicates p<0.05, \*\* indicates p<0.01 and ns indicates p>0.05.

**Figure 3. Effect of caspase-inhibitors on histamine-induced eosinophil apoptosis.** Percentage changes in apoptosis by histamine (100  $\mu$ M) in the presence and absence of IL-5 (1 pM) and different caspase-inhibitors. Apoptosis was analyzed by the relative DNA fragmentation assay. Each data point represents the mean  $\pm$  SEM of n=6 independent determinations using eosinophils from different donors. \* indicates p<0.05, \*\* indicates p<0.01 and ns indicates p>0.05 as compared with the respective control (1% DMSO for Z-Asp-CH<sub>2</sub>-DCB and 0.5% DMSO for the other caspase-inhibitors). Z-Asp-CH<sub>2</sub>-DCB, a pan-caspase inhibitor; Q-VD-Oph, a broad-spectrum inhibitor of caspases 1, 3, 8, 9, 10 and 12; Z-DQMD-FMK, a caspase 3 inhibitor; Z-VEID-FMK, a caspase 6 inhibitor; Ac-IETD-CHO, a caspase 8 inhibitor; Ac-LEHD-CHO, a caspase 9 inhibitor.

**Figure 4. Effect of histamine H<sub>1,3</sub> receptor antagonists on histamine-induced eosinophil apoptosis.** The apoptotic indices of eosinophils cultured with IL-5 (1 pM), with or without histamine (100 μM) and a combination of histamine H<sub>1,3</sub> receptor antagonists. Apoptosis was analyzed by the relative DNA fragmentation assay. Each data point represents the mean ± SEM of n=6 independent determinations using eosinophils from different donors. \*\* indicates p<0.01 as compared with the respective control.

**Figure 5. Effect of histamine H<sub>4</sub> receptor antagonists on histamine-induced eosinophil apoptosis.** A) The apoptotic indices of eosinophils cultured with different concentrations of thioperamide in the absence and presence of histamine and IL-5. B) The apoptotic indices of eosinophils cultured with different concentrations of JNJ7777120 in the absence and presence of histamine and IL-5. C) The apoptotic indices of eosinophils cultured with different concentrations of MMPCI in the absence and presence of histamine and IL-5. Apoptosis was analyzed by the relative DNA fragmentation assay. Each data point represents the mean ± SEM of n=6 independent determinations using eosinophils from different donors. \* indicates p<0.05 and \*\* indicates p<0.01 as compared with the respective control.

**Figure 6. Role of different signal transduction pathways in human eosinophil apoptosis.** A) The apoptotic indices of eosinophils cultured with pertussis toxin (PTX) in the absence and presence of histamine and IL-5. PTX disrupts G<sub>i/o</sub>-protein function. B) The apoptotic indices of eosinophils cultured with PKA-inhibitor Rp-8-CPT-cAMPS in the absence and presence of histamine and IL-5. C) The apoptotic indices of eosinophils cultured with PLC-inhibitors D609 or U73122 in the absence and presence of histamine and IL-5. Apoptosis was analyzed by the relative DNA fragmentation assay. Each data point represents the mean ± SEM of n=4-5 independent determinations using eosinophils from different donors. \*\*\* indicates p<0.001 as compared with the respective control.

Fig 1

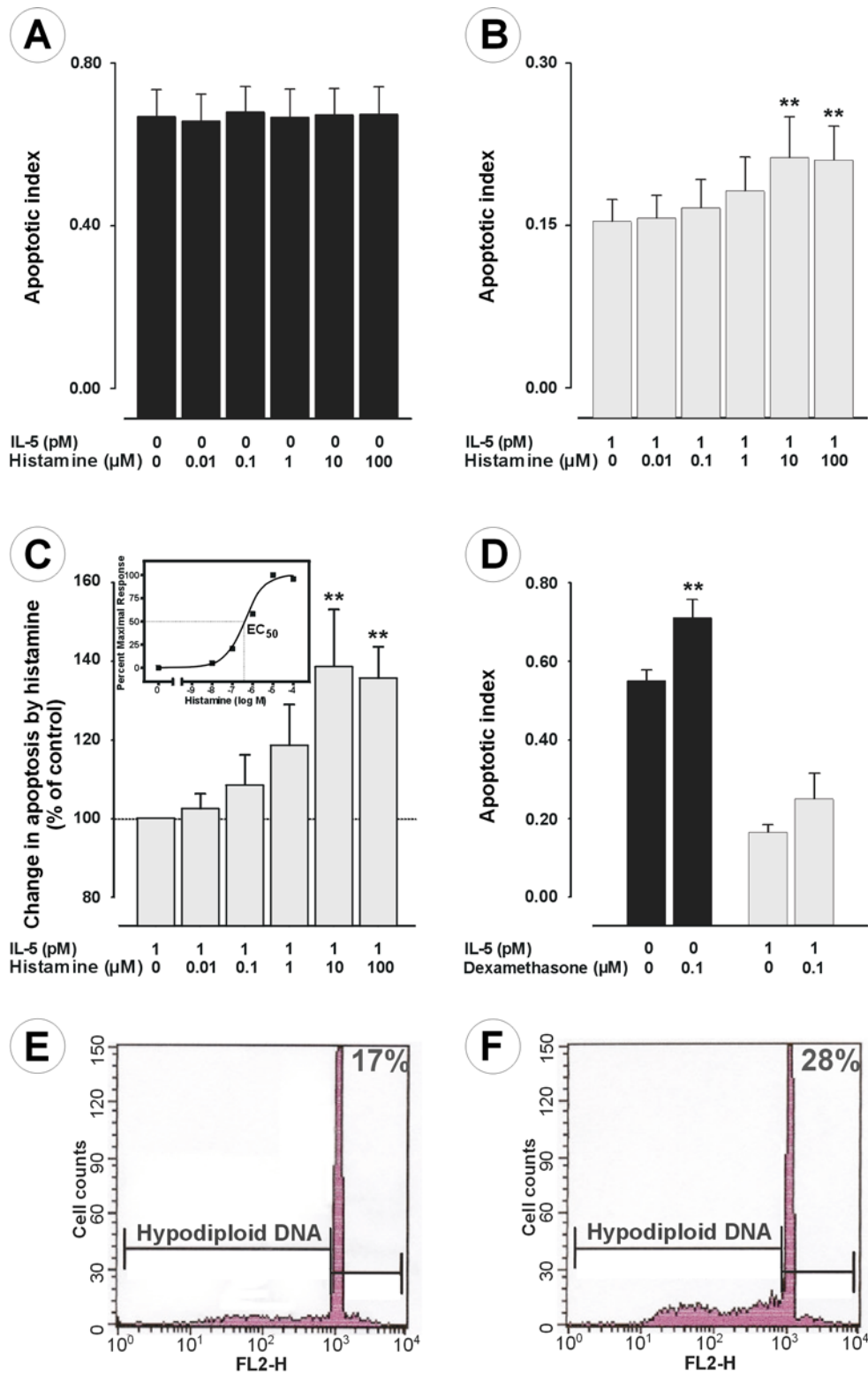
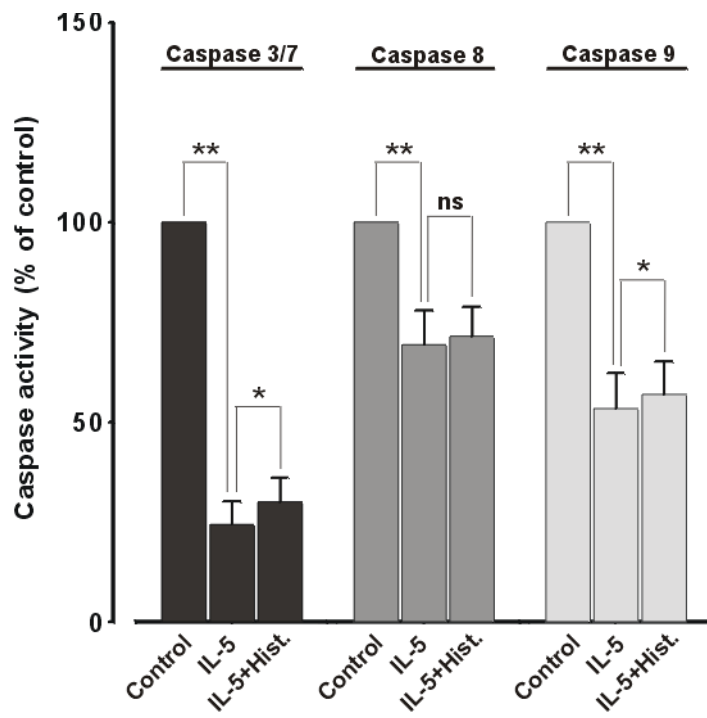
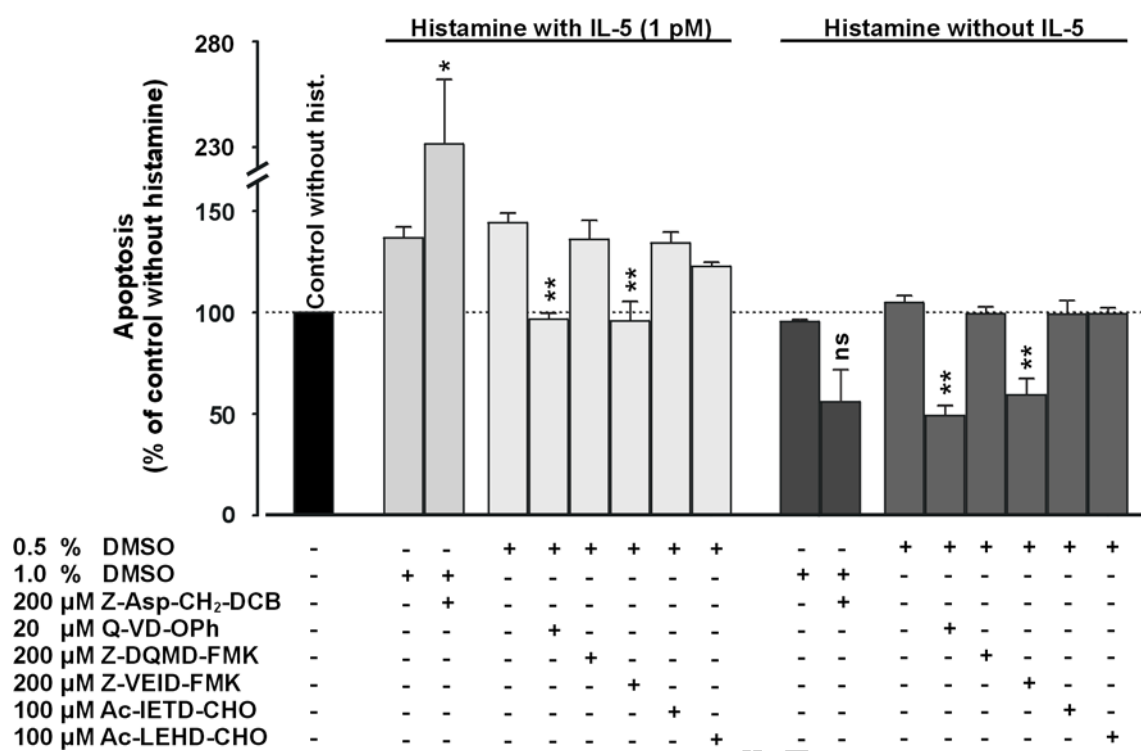


fig 2



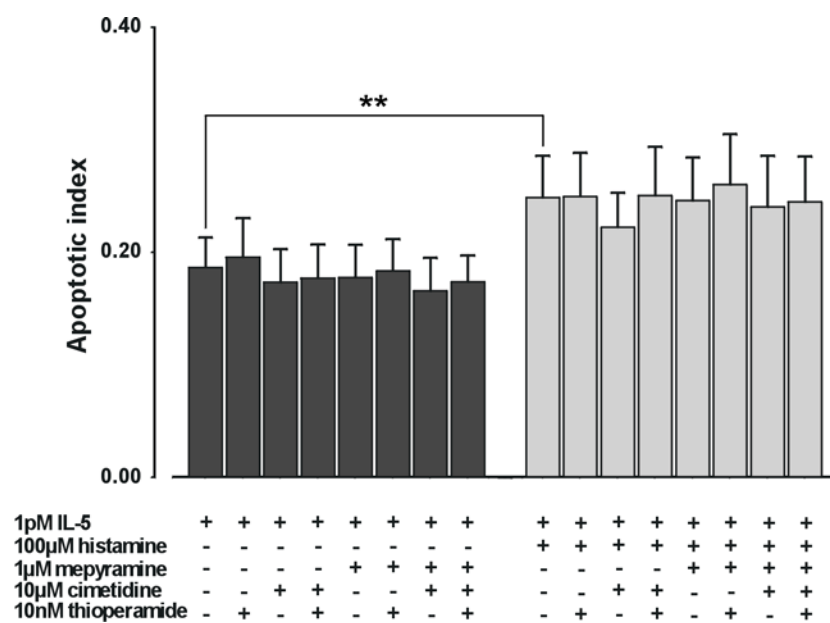
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fig 3



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fig 4



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fig 5

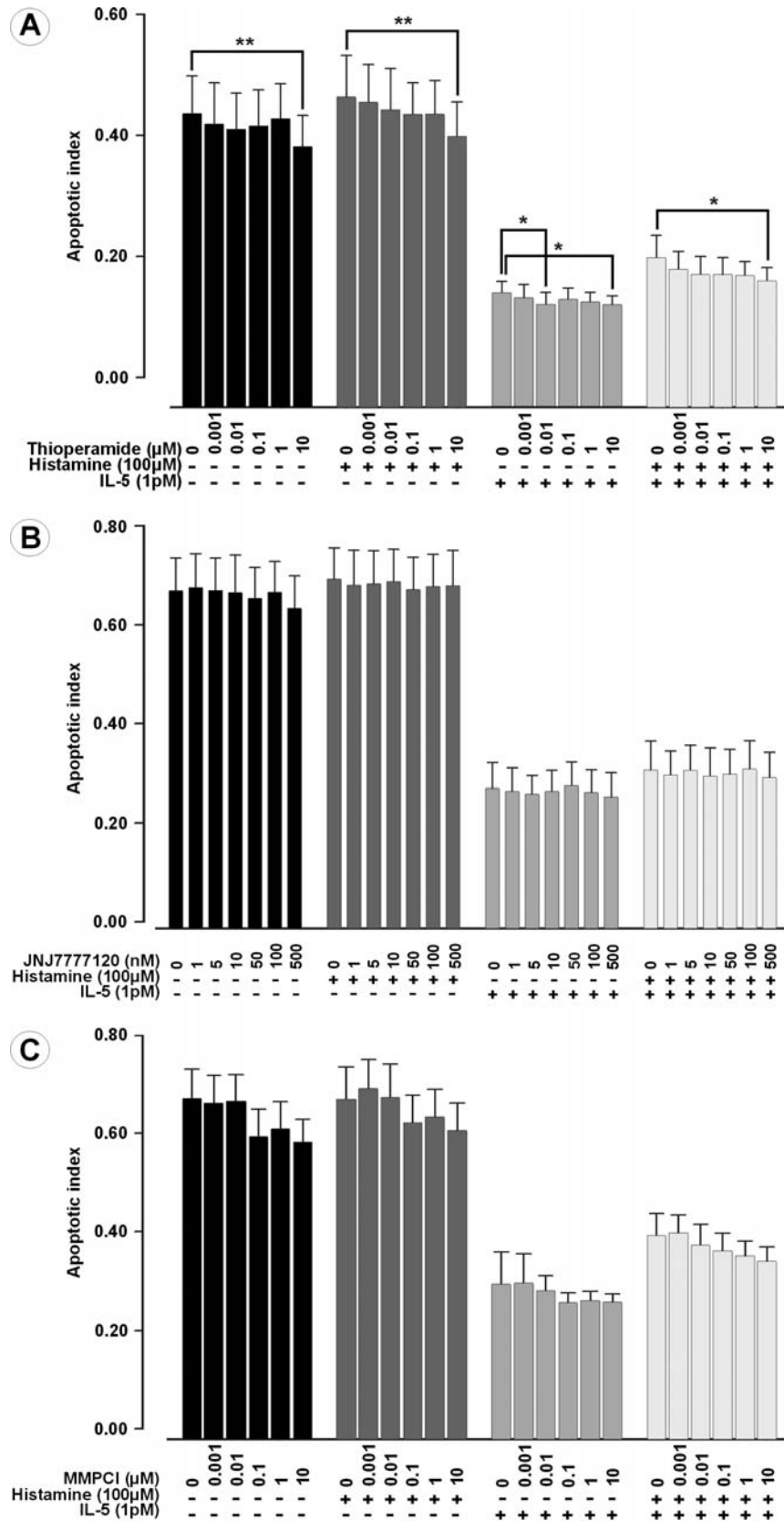


fig 6

