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Regulation of ERK1/2 and SAPK/JNK phosphorylation by histamine

By
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A THESIS

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Mitogen activated protein kinases (MAPKs) are specific serine/threonine kinases which respond to various stimuli and control various cellular activities including gene expression, mitosis, cell differentiation, and cell survival/apoptosis. Histamine is implicated in allergic disease and asthma and SAPK/JNK and ERK1/2 are involved in certain aspects of allergic inflammation such as TH2 differentiation and proliferation and apoptosis. This study was designed to investigate the effects of histamine on ERK1/2 and SAPK/JNK phosphorylation in splenocytes.

C57BL/6 splenocytes were treated with different concentrations of histamine ($10^{-4}$ M to $10^{-11}$ M). Histamine at higher concentration ($10^{-4}$ M) increased ERK2 phosphorylation. There was, however no significant effect seen at other concentrations ($10^{-6}$ M - $10^{-11}$ M). Surprisingly, H1 receptor agonist betahistine ($10^{-5}$ M), H2 agonist amthamine ($10^{-5}$ M), H3 agonist methimepip ($10^{-6}$ M) and H4 agonist 4-methyl histamine ($10^{-6}$ M), all increased ERK2 phosphorylation. H1R antagonist pyrilamine ($10^{-6}$ M), H2R antagonist ranitidine ($10^{-5}$ M), H3/H4R antagonist thioperamide ($10^{-6}$ M), and H3R antagonist clobenpropit ($10^{-5}$ M) inhibited histamine mediated ERK2 phosphorylation suggesting that all four histamine receptor subtypes played some role in this phosphorylation. Since TNF-alpha causes phosphorylation of ERK1/2, we investigated whether histamine acted via secretion of TNF-alpha to affect ERK1/2 phosphorylation. As a consequence, TNF-alpha knockout mice were used and we found that TNF-alpha was involved in ERK2 phosphorylation. There was complete inhibition of ERK2 phosphorylation by histamine via H2, H3 and H4 agonists, but effects of H1 agonist were inconclusive in TNF-alpha knockout splenocytes. This suggested that histamine indirectly affected the ERK2...
phosphorylation via its effects on the secretion of TNF-alpha and H1 receptor played a role in this process.

We performed similar experiments with stress activated protein kinases/c-jun N-terminal kinases (SAPK/JNK). C57BL/6 mice splenocytes were treated with different concentrations of histamine (10^{-4} M to 10^{-11} M), phorbol 12 myristate 13-acetate (PMA) was used as a positive control and phosphorylation of SAPK/JNK was determined. Histamine inhibited phosphorylation of SAPK/JNK at high concentrations (10^{-4} M-10^{-8} M) and had no effect on SAPK/JNK phosphorylation at lower concentrations (10^{-9} M-10^{-11} M). Histamine receptor specific agonists were used to identify the histamine receptors involved in the inhibition of SAPK/JNK phosphorylation. H1R agonist beta-histine (10^{-5} M) decreased the phosphorylation of SAPK/JNK. The decrease in SAPK/JNK phosphorylation by histamine was predominantly an H1 receptor effect. H2R agonist amphetamine (10^{-5} M) did not show any significant effect on SAPK/JNK phosphorylation. H3R agonist methimazole (10^{-6} M) and H4R agonist 4-methyl histamine (10^{-6} M), increased SAPK/JNK phosphorylation. H1R antagonist pyrilamine (10^{-6} M), H2R antagonist ranitidine (10^{-5} M), H3/H4R antagonist thioperamide (10^{-6} M), and clobenpropit (10^{-5} M), partially reversed the histamine mediated inhibition of SAPK/JNK phosphorylation. TNF-alpha knockout mice were used to determine if histamine regulated SAPK/JNK phosphorylation via TNF-alpha. In TNF-alpha knockout mice splenocytes, histamine inhibited SAPK/JNK phosphorylation. Activation of H1 receptors inhibited SAPK/JNK phosphorylation in knockout as was the case in wild type mice. Histamine via H2 receptor inhibited SAPK/JNK phosphorylation in knockout mice, but did not show any significant effect in wild type mice. Activation of H3 receptors
decreased SAPK/JNK phosphorylation in knockout mice, as opposed to an increase in wild type mice suggesting that another cytokine besides TNF-alpha was involved in SAPK/JNK phosphorylation. H4 receptor did not show any significant effect in knockout mice, but showed an increase in SAPK/JNK phosphorylation in wild type mice suggesting that TNF-alpha is required for histamine mediated effects via H4 receptors. This data showed a role of TNF-alpha in histamine-mediated effects of SAPK/JNK phosphorylation via H4 receptors.
PREFACE

Publications:

Radhika D Dandekar and Manzoor M Khan. Regulation of ERK2 phosphorylation by histamine in splenocytes. (Submitted)

Radhika D Dandekar and Manzoor M Khan. Regulation of SAPK/JNK phosphorylation by histamine in splenocytes. (Submitted)
DEDICATION

To my parents, Deepak and Savitri Dandekar
Brother Nihar Dandekar
Maternal grandparents Jagdish and Snehalata Godbole
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INTRODUCTION

1. Asthma:

Asthma is defined as a reversible airway obstruction disease manifested as wheezing, and caused by airway mucosa edema, inflammation, increased secretions, smooth muscle contraction and airway hyper reactivity. The signs of inflammation are edema, vasodilation, cellular infiltration, and pain. The inflammatory response in allergic asthma is characterized by excess production of immunoglobulin E, mast cell and eosinophil infiltration and activation in large and small airways, and increased levels of Th2 cytokines (IL-4, IL-5, IL-9, IL-13) by CD4+ lymphocytes. [1-4]

Asthma Pathogenesis:

The pathogenesis of asthma can be classified into inflammatory and remodeling components. Inflammatory component: The inflammatory response of asthma consists of a dense infiltrate in which eosinophils, mast cells, and CD4 helper T lymphocytes play an important role. Neutrophilic infiltration is also present in asthma exacerbations, and in the late response to allergen challenge. Dendritic cells are responsible for antigen presentation in asthma. Antigens then cross-link the IgE, and as a result, mast cells are activated and they degranulate. They are important in the acute airway responses to allergens and may also contribute to remodelling in chronic cases. Activated eosinophils are present in asthma, and they are thought to contribute to airway epithelial damage, by releasing products like eosinophil major basic protein. T helper cells are important in the airway inflammation. Helper T cells differentiate into Th1 and Th2 cells, which produce
cytokines and chemokines. Th1 cells produce IL-2 and interferon gamma, and Th2 cells produce interleukin 4, 5, 9 and 13. Th2 cells, via secretion of IL-4, stimulate IgE production from B lymphocytes. Inflammatory cells are recruited into the airways by chemokines, and they have a degree of selectivity in the cells they attract. These chemokines are produced by airway smooth muscle cells and airway epithelium. Inflammatory cells bind to adhesion molecules on bronchial vessel endothelium and go through a process of transmigration into the airway interstitium. [5-8]

Remodeling Component: Acute inflammatory diseases are normally cured with repair processes that restore the normal structure and function. This process becomes disturbed in asthma and ineffective repair leads to remodeling. When there is epithelial damage and protective barrier function loss, the deeper airway structures are exposed to environmental insults, and the inflammatory and structural cells produce growth factors which cause angiogenesis, smooth muscle proliferation in the airway, thickening of basement membranes, and fibrosis. The smooth muscle cell mass increases in the airways, and this causes bronchial responsiveness by increasing the force in response to bronchoconstrictor stimuli and there is reduction in the airway’s diameter. The cytokines and enzymes involved in the remodeling process are transforming growth factor, epidermal growth factor, and matrix metalloproteinases. [3]

The National Heart, Lung, and Blood Institute categorizes asthma severity into 4 categories: mild intermittent, mild persistent, moderate persistent and severe persistent asthma. The factors that can commonly cause asthma are allergens, upper respiratory tract infection, exercise, perfume and fumes, and temperature and humidity changes. [5, 8, 9, 10]
Biomarker of Airway Inflammation: Exhaled nitric oxide has been used as a means of indirectly measuring the inflammation in asthma. It is a biomarker of inflammation. Exhaled nitric oxide levels in asthma patients and healthy volunteers was measured to study peripheral blood lymphocyte cytokine expression, and also to study the relationship between exhaled nitric oxide and intracellular cytokine expression. Nitric oxide was elevated in patients with moderate to severe asthma and with treatment can be decreased in the first week reaching to a near normal level. Elevated exhaled nitric oxide is associated with decrease in the levels of IL-4 and IL-13 cytokine expression by CD8 lymphocytes. [11-12]

2. Cytokines in asthma:

The airway inflammation in asthma is regulated by a network of cytokines. The pathogenesis of asthma is a two step process. The first step is allergen sensitization, involving the development of antigen specific T-helper (Th2) cells. The second step, which does not occur in all atopic individuals, consists of targeting the Th2-driven allergic inflammation to the lower airways. This inflammatory process is regulated by a complex network of cytokines and growth factors, secreted by inflammatory cells and epithelial cells, fibroblasts and smooth muscle cells. The resulting inflammation of the airway mucosa shows acute as well as chronic type of inflammation. This latter phenomenon causes remodeling of the airway wall, resulting in a number of structural alterations. [13-15]

a] Th2 cytokines:
i] Interleukin-4:

Interleukin-4 shows a varied range of biological activities. It is the main cytokine involved in the pathogenesis of allergic disease. Additional effects of IL-4 which are important in asthma are stimulation of mucus production and fibroblasts. This implicates IL-4 in airway remodeling in asthma. Recombinant human IL-4 inhalation causes airway eosinophilia and bronchial hyper responsiveness in atopic asthmatics. Bronchial biopsy studies show that there was increased IL-4 expression of messenger ribonucleic acid (mRNA) and the protein level in the airway mucosa of atopic asthmatics compared to non asthmatic controls. IL-4 exerts its biological activities by binding to the IL-4 receptor (IL-4R), which is present in diverse cells. It is a heterodimer, consisting of the IL-4-binding IL-4R alpha chain and a second chain, which is either the cc chain (shared in common with the receptor for IL-2,-7, -9 and -15) or the IL-13R alpha chain. Increased IL-4R alpha expression in the epithelium and sub epithelium of asthmatic airways has been observed. IL-4 and IL-4R alpha gene polymorphism is related to asthma severity. The IL-4-589T allele, which has been associated with IL-4 gene expression increase, is a risk factor in life-threatening asthma. [13, 16]

This shows that the important role of IL-4 lies in its effect on Th2 cell development, rather than IgE synthesis induction and the subsequent mast cell degranulation. Exogenous administration or lung-specific over expression of IL-4 in transgenic mice causes epithelial hypertrophy and some peribronchial inflammatory change. But this is not followed by increased airway hyper responsiveness, which suggests that IL-4 in itself is not sufficient to induce all the changes characteristic of asthma. Other Th2-derived cytokines are also involved. An obvious possibility in this case is interleukin-5. [13, 16]
Eosinophils are key effectors in asthma pathogenesis. IL-5 is the primary cytokine involved in eosinophil production, differentiation, maturation and activation. IL-5 administered exogenously caused eosinophilia in a variety of in-vivo models. IL-5-expressing transgenic mice develop lifelong eosinophilia, whereas GM-CSF-expressing transgenic mice only a minimal increase in the number of eosinophils. It has been observed that, even in IL-5/- mice, a small number of morphologically normal eosinophils remain detectable in the blood stream. However, in the absence of IL-5, local injection of chemokines like eotaxin cannot cause eosinophilia, even though the donor eosinophils have been administered to restore or increase the circulating pool. IL-5 inhalation increases eosinophil percentage in induced sputum and airway hyperresponsiveness in asthma. Also, expression of IL-5 is increased at the mRNA and protein level in the mucosa of asthmatic airways. Expression of IL-5 mRNA has even been shown to correlate with clinical indices of disease severity. Passive transfer of IL-5-secreting CD4z T-cells from ovalbumin-sensitized wild-type mice to non sensitized IL5/- mice caused eosinophilia of airways and an increase in airway responsiveness when the animals were exposed to aerosolized ovalbumin. IL-5 reconstitution or IL-5 production, but not IgE, in IL-4/- mice restored the allergen-induced airway changes. This shows that IL-4 and IL-5 are involved sequentially in the pathogenesis of allergic airway changes. [13, 16]
Another cytokine which is derived from Th2 cells, and eosinophils, is interleukin-9. In vitro data has shown that IL-9 stimulates activated T-cell proliferation, increases IgE production from B-cells, promotes mast cells proliferation and differentiation, up regulates the alpha chain of the FceRI receptor and induces chemokine expression in lung epithelial cells. It also contributes to allergen induced airway changes. Human data showed that there was increased expression of IL-9 in bronchial biopsy samples of asthmatics. Genetic studies have also linked IL-9 to bronchial hyper responsiveness as a major characteristic of asthma. The lung-specific over expression in transgenic mice induces airway hyper responsiveness as well as morphological changes that are similar to asthma. However, IL-9 knockout mice experiments indicate that, although IL-9 may play a role in goblet cell hyperplasia and mast cell development, it did not have any substantial effect on eosinophils, T-cell development or immunoglobulin response. [13, 16]

iv] Interleukin-13

IL-13 is present in increased amounts in asthmatic airways, and its biological activities are similar to IL-4. The receptor IL-13R consists of the IL-13R alpha1 or alpha 2 chains, which binds to IL-13, and the IL-4R alpha chain, shared with the IL-4R. The signal transduction pathways are common to both receptors and they involve the intracytoplasmatic domain of both chains and are dependent on STAT-6. IL-13 can bind only to its own receptor. It has been hypothesized that, IL-4 is crucial for the initial Th2 development during primary sensitization and IL-13 release imperative during secondary antigen exposure. This is shown in a number of in vivo animal models. During secondary antigen exposure, IL-13 plays an important role than IL-4. In line with these observations, if endogenously released IL-13 was neutralized with an IL-13R alpha 2Fc
fusion protein during secondary antigen exposure, it characteristics of asthma in murine models were inhibited. [13, 16, 17]

b) Proinflammatory cytokines:

Another group of cytokines which are important are the pro-inflammatory cytokines such as TNF-alpha and IL-1beta. Tumor necrosis factor a (TNF-alpha) is an inflammatory cytokine produced by macrophages/monocytes and is released during acute inflammation. It is responsible for diverse signaling events in the cells, which can lead to necrosis or apoptosis. It plays an important role in infection resistance and cancer. TNF-alpha exerts its effects by binding as a trimer, to either a 55 kDa cell membrane receptor named as TNFR-1 or a 75 kDa cell membrane receptor termed TNFR-2. Both these receptors belong to the TNF receptor super family. This super family includes FAS, CD40 and CD27. These receptors have an extra cellular domain consisting of two to six repeats of cysteine rich motifs. Also, a number of structurally related “decoy receptors” exist which sequester TNF molecules, and rescue the cells from apoptosis. The pleiotropic activities of TNF-alpha include pro-inflammatory effects such as leukocyte recruitment through up regulation of adhesion molecules on vascular endothelial cells and induction of cytokine and chemokine synthesis. TNF-alpha also stimulates mesenchymal cells such as fibroblasts or smooth muscle cells, and plays an important role in the pathogenesis of airway remodeling. Increased levels of TNF-alpha have been detected in sputum, bronchoalveolar lavage fluid and biopsy samples from asthmatics. TNF-alpha inhalation causes airway hyper responsiveness and an increase in sputum neutrophil counts in healthy volunteers. TNF-alpha is important in determining the severity of asthma. [13, 18-21]
c] Immunomodulatory cytokines:

Interleukin-12, interleukin-18 and interferon gamma:

IL-12 expression is decreased in bronchial biopsy samples from asthmatic patients. It is produced by antigen-presenting cells plays an important role in Th1/Th2 differentiation in primary antigen presentation. The major cell involved in the sensitization process to allergens is the dendritic cell in the airway epithelium. Naive T-cells in the lymph nodes are stimulated and differentiate towards a Th1- or Th2- like response. IL-12 is a necessary cofactor for Th1 cell development. It induces interferon (IFN) production and down regulates IL-5 production, by an endogenous release of IL-10. In in-vivo animal models, IFN prevents antigen-induced airway eosinophilia development and hyper responsiveness. Similarly, IFN-R−/− mice develop airway eosinophilia in response to allergen. However, exogenous administration of IFN in humans has been disappointing. [13, 22]

3. Histamine:

Histamine is an immunomodulator and mediator of allergy and inflammation processes. It is also a chemical messenger and aminergic neurotransmitter, playing an important role in a multitude of physiological processes in the central nervous system and peripheral tissues. Mast cell histamine plays an important role in the pathogenesis of various allergic conditions, e. g., histamine release leads to various well-known symptoms of allergic conditions in the skin and the airway system. It is also implicated in tumor cell proliferation and promotion. e. g, in colon tumor, gastric tumor and melanoma, and via cytokine production in the tumor and in the tumor-surrounding environment. [22-25]
History:

Histamine (beta-aminoethylimidazole) was detected as a uterine stimulant in ergot extracts. It occurs in plant and animal tissues. It is a component of some venoms and stinging secretions. Histamine was first synthesized in 1907 and isolated from mammalian tissues. Histamine is implicated in immediate allergic and inflammatory reactions. It also plays a role in anaphylaxis as well as chemotaxis of white blood cells. It is involved in gastric acid secretion and is also a neurotransmitter and neuromodulator in central nervous system. Dale and Laidlaw discovered that histamine stimulated smooth muscle cells and had vasoconstrictor effect. They observed that histamine contributed to immediate hypersensitivity reactions and cellular injury. Best et al [26] isolated histamine from liver and lung, determining that the amine is a natural constituent in mammalian tissues. Therefore, histamine was named for the greek word histos meaning tissue. [26] Lewis and colleagues hypothesized that H-substance has histamine like properties and was released from skin cells by stimuli like antigen-antibody reactions. This showed that histamine played a role in allergic reactions and regulates gastric acid secretion. [26]

Chemistry:

Histamine is a hydrophilic molecule and it consists of an imidazole ring and an amino group connected by two methylene groups. [27] The pharmacologically active form at all histamine receptors is the monocationic N—H tautomer, i.e., the charged form of the species. [28] Different chemical properties are involved in interactions with H1 and H2 receptors. The histamine receptors: H1, H2, H3 and H4 are activated differently by histamine analogs. 2-methylhistamine responses are mediated by H1 receptors, whereas 4(5)-methyl histamine has an effect on H2 receptors. [29] A chiral analog of histamine
which has restricted conformational freedom, (R)-alpha-methylhistamine, is an H3 receptor agonist. It is also a weak agonist at H4 receptor. [30]

![Histamine molecule]

**Distribution:**

Histamine is distributed in many venoms, bacteria, and plants. Mammalian tissues contain histamine in amounts which range from less than 1 to more than 100 μg/g. Plasma concentrations are normally very low, but human cerebrospinal fluid contains a significant amount of histamine. The mast cell is the primary storage site for histamine in most tissues. Histamine concentration is high in mast cells present in the skin, bronchial and intestinal mucosa. [31]

**Pharmacokinetics:**

Histamine is decarboxylated by the amino acid L-histidine. It is a reaction which is catalyzed by the enzyme histidine decarboxylase in mammalian tissues. The chief histamine storage site tissue is the mast cell and in the blood, it is the basophil. Mast cells synthesize histamine and store it in secretory granules. Histamine is positively charged at the secretory granule pH of approximately 5.5, and it is complexed ionically with negatively charged acidic groups on other constituents of the secretory granule,
mainly proteases and heparin or chondroitin sulfate proteoglycans [32]. Histamine formations in non–mast cell sites are the epidermis, the gastric mucosa, and neurons in the central nervous system. There is a rapid turnover at these non–mast cell sites because the histamine is continuously released instead of being stored. Non–mast cell histamine site production contributes to the daily histamine excretion metabolites in the urine. Metabolism of histamine involves ring methylation to form N-methylhistamine, which is catalyzed by histamine-N-methyltransferase. Most of the N-methylhistamine formed is converted to N-methylimidazoleacetic acid by monoamine oxidase. Alternatively, histamine can also undergo oxidative deamination by the nonspecific enzyme diamine oxidase which yields imidazoleacetic acid, which undergoes conversion to imidazoleacetic acid riboside. These metabolites have no activity and they are excreted in the urine. Measurement of N-methylhistamine in urine gives an estimate of histamine production. Certain diseases like urticaria, gastric carcinoid, and sometimes myelogenous leukemia are associated with increased mast cells or basophils and there is increased excretion of histamine and its metabolites. [31, 32]

**Physiological roles of histamine:**

i] Allergic response:

The main target for immediate hypersensitivity reactions by histamine are mast cells and basophils. Immunoglobulin E (IgE) antibodies are generated in an allergic response to an allergen and they bind to mast cells and basophils surfaces through high-affinity Fc receptors specific to IgE. This receptor, FcεRI, consists of alpha, beta, and two gamma subunits. The IgE molecules are receptors for the antigens and they interact via FcεRI with signal-transduction systems in the cell membranes. The antigen forms the bridge
between the IgE molecules and activates signaling pathways in mast cells or basophils. These involve tyrosine kinases and cause subsequent phosphorylation of multiple protein substrates within five to fifteen seconds of contact with the antigen. These events trigger the exocytosis of secretory granules. [33, 34]

ii] Autacoid release:

Histamine release in hypersensitivity reactions also results in the release of other inflammatory mediators. IgE receptor stimulation also activates phospholipase A₂ (PLA₂), which produces a host of other mediators, such as the platelet-activating factor and arachidonic acid metabolites. Leukotriene D₄, generated in this manner, is a potent smooth muscle contractor of the bronchial tree. Kinins are also released during some allergic responses. Therefore, the mast cell secretes varied inflammatory mediators in addition to histamine, which are responsible for the major symptoms in the allergic response. [31]

iii] Release of mediators:

Considerable importance has been designated to the regulation of mediator release from mast cells and basophils. These cells contain receptors connected to signaling systems which can enhance or inhibit the IgE-induced mediator release. Agents acting at muscarinic or adrenergic receptors increase the release of mediators, but it is not clinically significant. Epinephrine acting through beta2 adrenergic receptor increases cellular cyclic AMP and inhibits the secretory activities of mast cells. The positive effects of beta adrenergic agonists in asthma are due to their ability to relax bronchial smooth muscles. Cromolyn sodium inhibits the release of mediators from mast and other cells in the lung. [31]
iv] Triple response:

Histamine, when injected intradermally, causes a distinctive red spot, edema, and a flare response. This effect involves three different cell types: smooth muscle in the microcirculation, capillary endothelium, and sensory nerve endings. A reddening appears at the site of injection due to blood vessel dilation, subsequently followed by an edematous wheal at the site of injection and a red irregular flare which surrounds the wheal. The flare is caused by an axon reflex. An itching sensation can accompany these effects. Similar local effects are observed when injecting histamine liberators (compound 48/80, morphine) intradermally or by an application of antigens to the skin of a sensitized person. These effects are prevented by administering an H1-receptor-blocking agent, although H2 and H3 receptors can also be used. [31]

v] Gastric acid secretion:

Histamine, via H2 receptors, increases gastric acid secretion from parietal cells. It also increases pepsin and intrinsic factor output. Gastric acid secretion is also increased by vagus nerve stimulation and by the hormone gastrin, via M3 and CCK2 receptors activation on the parietal cell. Acetylcholine and gastrin also stimulate release of histamine from the enterochromaffin like cell. Histamine is the principal physiological mediator of acid secretion: H2 receptor blockade decreases acid secretion in response to histamine and also causes inhibition of responses to gastrin as well as vagal stimulation. [31]

vi] Central nervous system:
Histamine is a potent neurotransmitter in the central nervous system. Histamine, histidine decarboxylase, and enzymes which catalyze histamine degradation are distributed in the central nervous system and are mainly present in synaptosomal fractions of brain homogenates. H1 receptors are ubiquitous in the central nervous system and are densely concentrated in the hypothalamus. Histamine increases wake cycle via H1 receptors. Histamine through H1 receptors also causes appetite inhibition. Histamine-containing neurons may be involved in the regulation of drinking, body temperature, and the secretion of antidiuretic hormone, and in blood pressure control and pain perception. H1 and H2 receptors, both are involved in these responses. [34] H1 receptor knockout mice caused increased aggression, problems with locomotion, and other neurological symptoms. [35] Central effects of histamine are mediated by H3 autoreceptors, present exclusively in the brain. This information is consistent with anxiety and cognition changes in animals treated with H3 receptor antagonists. [36, 37]

Cardiovascular System:

Histamine injections or infusions result in systolic and diastolic blood pressure decrease and an increase in heart rate. These changes are caused by the vasodilator action of histamine on arterioles and precapillary sphincters. Histamine acts as a stimulant on the heart and causes reflex tachycardia. Flushing, a sensation of warmth, and headache can occur during administration of histamine which is consistent with the vasodilatation. [31] Vasodilatation is caused by activation of the H1 receptor and is mediated by nitric oxide release from the endothelium. Blood pressure decrease is followed by reflex tachycardia. Histamine at higher doses activates the H2-receptor mediated cAMP vasodilatation and direct cardiac stimulation. These cardiovascular effects of small doses of histamine can
be blocked by H1 receptor antagonists alone. Histamine-induced edema is caused by H1 receptors in the post capillary vessels. This is responsible for urticaria, which releases histamine in the skin. Histamine increases contractility and increases pacemaker rate. These effects are mediated by H2 receptors. Histamine decreases contractility in the atrial muscles and this effect is mediated by H1 receptors. [31]

viii) Bronchi:

Histamine causes bronchoconstriction which is mediated by H1 receptors. Bronchoconstriction after small doses of histamine is not significant. However, asthma patients are very sensitive to histamine. The bronchoconstriction in these patients probably depicts a hyperactive neural response, as such patients also respond to many other stimuli, and this response to histamine can be blocked by ganglionic blocking agents as well as by H1 receptor antagonists. Tests using small doses of inhaled histamine are used in the diagnosis of bronchial hyper reactivity in patients with suspected asthma or cystic fibrosis. These individuals may be very sensitive to histamine than normal patients. [31] The effects of histamine are mediated through four pharmacologically distinct receptors, i.e., the H1, H2, H3, and H4 receptors, are all members of the G-protein coupled receptor (GPCR) family. [22-25, 38-41]

3.1 H1 receptor:

Distribution and function:

The human H1 receptor (H1R) is present on the third chromosome (3p21-p14), composed of 487 amino acids and has ~75–85% interspecies homology. In vascular endothelial cells, histamine H1-receptor stimulation causes cellular responses like changes in
vascular permeability due to endothelial cell contraction, prostacyclin synthesis, synthesis of platelet-activating, release of Von Willebrand factor and nitric oxide release. The H1-receptor is present on human T lymphocytes and it also increases intracellular calcium. They are present in the adrenal medulla and release catecholamines. Histamine releases adrenaline and nor adrenaline from cultured bovine adrenal chromaffin cells. Histamine can also cause the release of leucine- and methionine-enkephalin. After prolonged exposure to histamine, there is an increase in messenger ribonucleic acid-encoding proenkephalin A. In human atrial myocardium, histamine produces negative inotropic effects. H1-receptors are distributed widely in the mammalian brain. Higher densities of H1-receptors are found in neocortex, hippocampus, nucleus accumbens, thalamus, and posterior hypothalamus, whereas cerebellum and basal ganglia show lower densities of histamine. There is inhibition of firing and hyper polarization in hippocampal neurons after H1-receptor activation. However, hypothalamic supraoptic, brainstem and human cortical neurons are excited by histamine H1-receptor activation via blocking the potassium conductance. [22-25, 42-44]

Signal transduction:

The H1 receptor preferably couples to the $G_{q/11}$ family of G-proteins and causes mobilization of intracellular Ca$^{++}$. Histamine activates phospholipase C (PLC), which mediates phosphatidyl inositol diphosphate (PIP2) activation, resulting in the formation of inositol triphosphate (IP$_3$) and 1, 2-diacyl glycerol (DAG). IP$_3$ mediates Ca$^{++}$ release from endoplasmic reticulum, and also increases Ca$^{++}$ influx from the extracellular space as a secondary but longer lasting event. Due to Ca$^{++}$ ion influx, a secondary breakdown of membrane phosphoinositides occurs. IP$_3$ can be phosphorylated to produce IP$_4$ which
further increases the intracellular Ca\(^{++}\) level. DAG activates a serine/threonine kinase, the protein kinase C (PKC) which phosphorylates and activates other proteins. DAG can also stimulate another enzyme, called phospholipase A2 (PLA2), which forms arachidonic acid metabolites, such as prostaglandin E2, prostacyclin, and thromboxane A2. In addition to the above effects, activation of H1 receptor leads to activation of several other signaling pathways, which are secondary to changes in intracellular calcium concentration or protein kinase C activation. Histamine can stimulate nitric oxide synthase activity via a Ca\(^{++}\)/calmodulin-dependent pathway and causes activation of soluble guanylyl cyclase in different cell types. Arachidonic acid release and the synthesis of arachidonic acid metabolites such as prostacyclin and thromboxane A2 is increased by H1-receptor stimulation. In most of the tissues, histamine H1-receptor activation does not activate adenylyl cyclase directly, but amplifies the direct cAMP responses to histamine H2-, adenosine A2- and vasoactive intestinal polypeptide receptors. [22-25, 42-44]

3.2 H2 receptor:

Distribution and function:

H2 receptor is involved in the relaxation of airway and vascular smooth muscle, regulation of right atrial and ventricular muscle of the heart, inhibition of basophil chemotactic responsiveness, various actions on immune cells and inhibition of prostaglandin E2-stimulated duodenal epithelial bicarbonate secretion. Histamine H2-receptors have a potent effect on secretion of gastric acid. High histamine concentrations are also present in cardiac tissues of most animal species and can cause positive chronotropic and inotropic effects on atrial or ventricular tissues via H2-receptor
stimulation. H2-receptor–mediated smooth muscle relaxation is seen in airway, uterine, and vascular smooth muscles. Histamine H2- receptors inhibit a variety of functions within the immune system. H2-receptors on basophils and mast cells negatively regulate histamine the release. Also, H2-receptors inhibit antibody synthesis, T-cell proliferation, cell-mediated cytolysis, and cytokine production on lymphocytes. In the central nervous system, H2-receptor activation inhibits nerve cells, and blockade of long-lasting after-hyper polarization and the accommodation of firing, which leads to potentiation of excitation in rodents and human brain. A slow excitation is also common. Synaptic transmission in the hippocampus is increased, and synaptic plasticity is enhanced. [22-25, 42-44]

Signal transduction:

The histamine H2 receptors couple to adenyl cyclase via the Gs protein, and histamine stimulates cAMP production in many different cell types, e. g., the CNS and CNS-derived cells, gastric mucosa, cardiac myocytes, fat cells, vascular smooth cells, basophils. Increased cAMP concentration activates protein kinase A (PKA), phosphorylating a wide variety of proteins in the cells however, the H2R signaling pathway has a dual face. In addition to the adenyl cyclase-mediated one, histamine through H2 receptor also increases the intracellular Ca++ ion level in some cell types, e. g., gastric parietal cells and HL-60 leukemic cell line. Activation of the Gs-cAMP-PKA pathway promotes Ca++ influx through phosphorylation of L-type Ca++ channels. In addition to Gs-coupling to adenylyl cyclase, H2-receptors couple to other signaling pathways. For example, in gastric parietal cells, H2-receptor stimulation increases intracellular free concentration of calcium ions. The responses to H2-receptor–stimulated
calcium and inositol trisphosphate were both inhibited by cholera toxin treatment. H2-receptors release calcium from intracellular calcium stores in parietal cells. [22-25, 42-44]

### 3.3 H3 receptor:

Distribution and function:

Histamine H3-receptors are involved in inhibiting neurotransmitter release. The H3-receptor was first discovered as an autoreceptor which regulates histamine synthesis and release from rat cerebral cortex, striatum, and hippocampus. H3-receptor–mediated inhibition of histamine release is seen in human cerebral cortex. H3-receptors can regulate serotonergic, noradrenergic, cholinergic, and dopaminergic neurotransmitter release. These receptors regulate the release of sympathetic neurotransmitters in guinea pig mesenteric artery, human saphenous vein guinea pig atria, and human heart. Inhibition of parasympathetic nerve activity has also been observed in guinea pig ileum and human bronchi and trachealis. H3 receptors are involved in neuropeptide release (tachykinins or calcitonin gene-related peptide) from sensory C fibers. [22-25, 42-44]

Signal transduction:

In cells transfected with the human H3 receptor, histamine and R-α-methyl histamine could inhibit forskolin-stimulated cAMP production, and this inhibition is abolished by pretreatment of the cells with PTX, indicating that the receptor is coupled to Gi/o G-proteins. The receptor can also signal via increases in intracellular calcium, observed when the mouse receptor is cotransfected with chimeric Gq15 G-proteins. Furthermore, it has been shown that activation of rat H3 receptor can increase p44/p42 MAP kinase
phosphorylation and arachidonic acid release. It has a high degree of constitutive activity; that is, activity in the absence of agonists. This opens up the possibility that H3 receptor ligands that were initially characterized as antagonists may in fact be inverse agonists, i.e., they inhibit the constitutive activity of the receptor. [22-25, 42-47]

3.4 H4 receptor:

Distribution and function:

The H4 receptor is present in the peripheral blood leukocytes, spleen, thymus, small intestine, colon and the bone marrow. H4 receptor functions as an immune modulator. A lot of additional work is needed for characterization of the H4 receptor. [22, 23, 25, 48]

Signal transduction:

The H4 receptor has been mapped to chromosome 18q11.2 and has a similar genomic structure as the H3 receptor. In transfected cells, the H4 receptor can inhibit forskolin-stimulated cAMP. The effect on cAMP can be blocked by pretreating the cells with PTX, suggesting a role for Gαi/o proteins. Activation can also lead to increases in intracellular calcium. There has been one report of activation of MAP kinases in transfected HEK-293 cells. Raible et al showed that histamine causes calcium mobilization in human eosinophils and this effect is not mediated by H1, H2, or H3 receptors, involving the H4 receptor. In primary cells, activation of H4R leads to increases in intracellular calcium, which is mediated by Gαi/o proteins and phospholipase C. Phospholipase C is activated by the released Gβγ subunits, and hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to diacylglycerol and inositol-1, 4, 5-triphosphate (IP3), causing the release of calcium from intracellular stores such as the
endoplasmic reticulum. The figure 1 below gives a detailed overview of the different histamine receptors. [22, 23, 25, 48]

Figure 1: Histamine receptors:

<table>
<thead>
<tr>
<th>Type of Receptor</th>
<th>Location</th>
<th>Response</th>
<th>Agonist</th>
<th>Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine H1</td>
<td>Smooth muscle, Endothelial cells, heart</td>
<td>Smooth muscle contraction, increased vascular permeability, hormone release, stimulation of NO formation</td>
<td>Histamine, Betahistine</td>
<td>Pyrilamine, mepyramine</td>
</tr>
<tr>
<td>Histamine H2</td>
<td>Gastric mucosa, neutrophils, vascular smooth muscles</td>
<td>Gastric acid secretion, smooth muscle relaxation, stimulation of adenylyl cyclase, positive chronotropic and ionotropic effect on cardiac muscle</td>
<td>Histamine, amthamine</td>
<td>Cimetidine, ranitidine</td>
</tr>
<tr>
<td>Histamine H3</td>
<td>CNS, heart, lung, endothelium</td>
<td>Neurotransmitter release inhibition, inhibition of gastric acid secretion</td>
<td>Histamine, Methimepip, imetit</td>
<td>Thioperamide, burimamide</td>
</tr>
<tr>
<td>Histamine H4</td>
<td>Bone marrow, white blood cells, thymus</td>
<td>Mast cell chemotaxis, immune modulator</td>
<td>Histamine, 4-methylhistamine</td>
<td>Thioperamide</td>
</tr>
</tbody>
</table>

3.5 Immune regulation by histamine:

Histamine has immune and inflammatory cell functions. Histamine recruits cells and is responsible for their maturation, activation, polarization and other functions which cause
inflammation. It also regulates B cells, T cells, monocytes and dendrites. Histamine is a chemoattractant too, which causes agonist induced polymerization, intracellular calcium mobilization, cell shape alteration and increase in adhesion molecule expression. Histamine increases the secretion of proinflammatory cytokines such as IL-1alpha, IL-1beta, IL-6 and IL-8 in allergic responses. It inhibits the production of IL-1, TNF-alpha, IL-12 and IL-18 activity in monocytes, but increases IL-10 secretion via H2 receptor stimulation. Histamine also down regulates CD14 activity via H2 receptors. H1 and H3 receptor stimulation can cause an intracellular calcium reflux, actin polymerization and chemotaxis. A dose of histamine increases cAMP and IL-12 levels in a dose dependent manner via H2 receptor. Histamine has an effect on the life span of immune cells too. It affects the monocyte life span via H2 receptor. It prevents apoptosis of monocytes in a dose dependent manner which is also mediated by the cAMP pathway. It has been seen that there is differential histamine receptor expression on Th1 and Th2 cells. Th1 cells show an expression of the histamine H1 receptor, whereas Th2 cells show increased expression of H2 receptors. H1 receptor knockout mice show suppression of IFN-gamma and increased secretion of Th2 cytokines: IL-4 and IL-13. H2 receptor knockout mice show an increase in both Th1 as well as Th2 cytokines. Stimulation of IL-3 increases H1 receptor expression on Th1 cells, but not Th2 cells. It is also seen that histamine stimulation causes IL-10 secretion via H2 receptor. This increased IL-10 secretion in T cells and dendritic cells may have an important implication in inflammatory functions by histamine. [49-53, 42, 43, 54-56]

4. MAPK: Mitogen Activated Protein Kinases:
They are specific serine/threonine kinases which respond to extra cellular stimuli called mitogens and regulate cellular activities like gene expression, mitosis, cell differentiation, and cell survival/apoptosis. It is composed of three consecutive kinases, (MAPKKK, MAPKK, and MAPK). MAPKKK (Raf-1, B-Raf) phosphorylates and activates MAPKK (MEK 1 and MEK2) at two serine residues which in turn phosphorylates and activates MAPK. It is initiated by a signal coming from outside the cell. [57-60]

MAPK Families:

There are four major groups identified in mammalian cells which are:

1] Extra cellular signal regulated kinase (ERK1 and ERK2)

2] Stress activated protein kinases/C-jun amino terminal kinase (SAPK/JNK)

3] p-38 kinase

4] Extra cellular signal regulated kinase 5 (ERK 5)
4.1 ERK1/2:

They are extra cellular signal regulated kinases and are activated by phorbol esters, growth factors and serum. They are involved in cell proliferation, cell differentiation and survival. ERK is activated by dual Tyr and Thr phosphorylation. It is activated at Thr202/Tyr204 for ERK1 and Thr185/Tyr187 for ERK2.

The extracellular signal-regulated kinase-1 (ERK1) and ERK2 is collectively called the Ras-Raf-ERK signal transduction cascade. When the ERK phosphorylation cascade is activated, it is linked to cell surface receptor tyrosine kinases (RTKs) and other upstream signaling proteins. [57]

RTKs:

The RTK family has intrinsic tyrosine kinase activity, and it catalyzes the transfer of the phosphate group of ATP to the hydroxyl groups of tyrosines on target proteins. All RTKs
have a extracellular ligand binding domain, connected through a transmembrane helix to the cytoplasmic domain. This domain contains a protein tyrosine kinase (PTK) core. The epidermal growth factor receptor was the first RTK to be discovered. The EGF receptor has nine tyrosine residues which can phosphorylate in the cytoplasmic domain. RTKs can aid in the recognition and recruitment of a specific complement of signaling proteins. One such protein is growth factor receptor bound protein 2 (Grb2). Grb2 has a central SH2 domain flanked by two Src homology 3 (SH3) due to which it can associate with the proline-rich regions of the nucleotide exchange factor son of sevenless (SOS). Grb2 recruitment from the cytoplasm to the plasma membrane brings SOS near the membrane-bound c-ras. SOS enhances GDP release and binding of GTP to Ras, converting this GTPase into its active form. [57, 61-63]

Ras:

Ras is a member of the family of GTPases. They are proteins which bind and hydrolyze GTP. Mutations of the Ras isoforms, which impair GTPase activity and stabilize the GTP bound state, are found in nearly one-third of all human cancers. Ras is anchored to the cytoplasm of the plasma membrane. This brings it into close proximity with SOS, and it stimulates the exchange of GDP bound to Ras with GTP from the cytosol. Ras is activated and it interacts with a number of downstream effectors. In the ERK signaling cascade, Ras is an adaptor which binds to effector Raf kinase with high affinity, and causes it to translocate to the cell membrane, where Raf is activated. [57, 61-63]

Raf:

Raf is a Serine/Threonine protein kinase and it catalyzes the phosphorylation of hydroxyl groups on specific Ser and Thr residues. It is present almost everywhere and the most
studied is Raf-1. Raf is activated by recruitment to the plasma membrane by GTP-bound as. The effector domain of Ras binds Raf at two locations in the MAP3K's N-terminus, the Ras-binding domain and the cysteine-rich domain. [57, 61-63]

MEK:

Phosphorylated Raf activates MAPK/ERK kinase 1 (MEK1) and (MEK2). They are also called as M KK1 and M KK2. Both MEKs are present everywhere in mammalian cells. Activation of MEK1 and MEK2 occurs through phosphorylation of two serine residues. Different Raf isoforms activate MEK1 and MEK2 differentially. Raf-1 contains two separate MEK binding sites, after phosphorylation of Raf-1 at ser338. The ERK cascade is implicated in a wide variety of human cancers. Many drugs are being developed which are in clinical trials for EGFR inhibition. Several Ras inhibitors have reached Phase I to III trials. Also, a number of kinase inhibitors against Raf and MEK have entered clinical trials. [57, 61-63]
4.2 SAPK/JNK:

The first member of the JNK family was isolated from rat liver. JNK1, JNK2, JNK3 are the three types of kinases. JNK3 is selectively expressed in neuronal tissues, while JNK1 and 2 are expressed everywhere. SAPK is activated by its specific MAPKinases, namely SEK1(MKK4) and SEK 2(MKK7). JNKs are also activated in response to cytokines, stress, UV radiation, growth factor deprivation. [57, 61-65]

The SAPK/JNK stress pathway takes part in intracellular signaling pathways which control cellular processes like cell proliferation, differentiation, transformation, apoptosis, migration, and cytoskeletal integrity. SAPK/JNK also phosphorylates transcription factors in addition to c-Jun, such as ATF-2, Elk-1, p53, and c-Myc, as well as nontranscription factors such as Bcl-2, Bcl-xL, paxillin, and MAP2. [57, 61-65]

SAPK/JNK is activated by the dual phosphorylation of Tyr and Thr residues which is located in a Thr-Pro-Tyr motif. It catalyzed by the dual specificity kinases, SEK1 and MKK7, which can catalyze phosphorylation of both threonine and tyrosine residues.

SAPK/JNK pathway either protects or increases sensitivity to apoptosis depending on the cell type, stimuli, and the latency of the MAPK activation. [57, 61-65]
4.3 p38:

There are four known p-38 isoforms (alpha, beta, gamma and delta) and they are associated with cell differentiation, inflammation, cell growth and cell death. It plays a central role in a wide variety of immunological responses. p38 gamma is present in skeletal muscles whereas p38 delta is found in lungs, kidney, intestine, pancreas and small intestine. The p38 pathway is activated by a variety of cytokines (IL-1, 2, 7, 17, 18) and TNF-alpha. MKK3, MKK4, MKK6 are the upstream kinases in p38 activation. Specific phosphatases down regulate p38. The downstream targets are other kinases or transcription factors like ATF-2 and MEF2. The main response of p38 is to activate inflammatory mediators to initiate leukocyte recruitment and activation. [57, 61-63]
4.4 ERK 5:

ERK5 is the fourth and least studied MAPKinas. It is called as extracellular regulated kinase 5 or also known as big MAPK 1 or BMK 1. It is activated in vivo in response to stress, growth factors like EGF (epidermal growth Factor), NGF (nerve growth factor), GPCRs (G-protein coupled receptors), neurotrophins or neurotrophic factors which induce the survival of neurons, Phorbol esters, oxidative and osmotic shock. [66] ERK 5 contains a dual phosphorylation motif consisting of Thr-Xaa-Tyr (TEY). It has a molecular mass of approximately 100kDa, a large C terminus which distinguishes it from ERK 1/2. Ras, MEKK2, MEKK3, MEk5 are its upstream components. Its downstream components are RSK, transcription factors: MEF2A, MEF2C. (Myocyte enhancer factor) Mitogens like epidermal growth factor and granulocyte colony stimulating factor transmit their signals via ERK5. EGF induced activation of this kinase can also be mediated via c
Src and Ras. Activation of ERK5 by G-CSF is regulated by protein tyrosine kinases and protein kinase C. [66] ERK5 is also activated by neurotrophins like BDNF, NGF and NT3/4 (neurotrophins 3/4). NGF activates ERK 5 via MEKK3 and MEK5, but BDNF activates ERK 5 by MEK5. It is also necessary for the survival response of the dorsal root ganglion to the neuronal growth factor. Tyrosine Kinase Receptor-A receptors are present on the surface of the axon and they autophosphorylate after NGF binds. These phosphorylated receptors get transported from the axon to the cell body and ERK 5 is activated. A phosphorylation cascade results and the transcription factor CREB (Ca\(^{++}\)/cAMP response element binding protein) is activated. CREB is not directly phosphorylated by ERK 5, but travels into the nucleus and phosphorylate the kinase RSK, which phosphorylates CREB.

Another factor which activates ERK 5 is stress. Sorbitol, H\(_2\)O\(_2\) (Hydrogen peroxide) and UV irradiations are some of the examples. H\(_2\)O\(_2\) stimulates ERK5 via c-Src tyrosine kinase and it increases MEF2C. After ERK 5 is activated, it phosphorylates various nuclear and cytoplasmic targets. MEF2A, MEF2C, and MEF2D are the transcription factors of the MEF family and they are substrates for ERK 5. ERK 5 induced phosphorylation of 2C increases transcription and there is increase in c-Jun gene expression. MEF2D is also a specific substrate for ERK5, but p38 MAPKs and ERK5 both control the activities of MEF2A and MEF2C. The ERK 5 cascade is implicated in cancer and heart disease and hence it is of great importance for new therapeutic strategies for diseases which are resistant to current therapies. [66, 67]

4.5 Mitogen activated protein kinases and immune response:
Various types of cells are involved in a number of immune responses which function as initiators, regulators, and effectors. These cells interact with each other to mediate gene expression. The MAP kinase cascade is a signaling pathway, which is involved in immune responses and in lymphocyte development. [68] The innate system, by definition is a basic resistance to the disease by a body. In mammalian systems, 10 Toll-like receptors (TLR) exist. Toll-like receptors (TLRs) are a class of single membrane, non-catalytic receptors. They play an important role in innate immunity. TLRs recognize “pattern” molecules on microbes which induce inflammatory responses. For example, TLR4 is important for LPS (lipopolysaccharide) recognition. TLR cytoplasmic domains resemble that of the interleukin 1 (IL-1) receptor, and therefore they are called Toll-IL-1-Receptor (TIR) domains. After ligand-mediated dimerisation, TLR recruits an adaptor protein called MyD88. MyD88 then assembles a signal containing IRAK, TRAF6, and ECSIT. These activate NF-kB and MAP kinases like p38 and JNK, which produces inflammatory cytokines like tumor necrosis factor (TNF)-alpha, IL-1, and IL-12. [68] p38 MAP kinases are activated by inflammatory cytokines like TNF and IL-1 and have specific functions in cellular responses to these cytokines. TNF-alpha and not IL-1 selectively inhibited p38 activation in mouse embryonic fibroblasts. Due to this deficiency, mouse failed to upregulate IL-1 and TNF-alpha. This showed that MKK3-p38 played a role in response to TNF, but not to IL-1. [68]

Janus kinases (JNK) activity can be induced by liposaccharides (LPS) or inflammatory cytokines like as TNF and IL-1. JNK is also activated by inflammatory cytokines and double stranded viral RNA in fibroblasts. Chu et al showed that JNK is required for production of type I Interferon and IL-6. [68]
4.6 Mitogen activated protein kinases in T helper cells:

CD4+ helper T cells play an important role in immune responses. Like cytotoxic T cells, Helper T recognize specific MHC-peptide complexes on antigen-presenting cells (APC). After they receive these signals, Th cells are triggered to produce interleukin 2 (IL-2) and they enter the cell cycle. After cell division, T cells differentiate into effector cells. Effector Th1 cells produce pro-inflammatory cytokines such as interferon-gamma and lymphotoxin-alpha. Th1 cytokine production is involved in organ-specific autoimmune diseases, including rheumatoid arthritis and insulin-dependent diabetes mellitus. Effector Th2 cells produce different cytokines like IL-4, IL-5, IL-9, IL-10, IL-13, and they instruct the B cells to proliferate and differentiate. Th2 cells provide protection against pathogens like bacteria and other parasites, and they are also involved in asthmatic reactions. Differentiation of naïve Th cells into Th1 or Th2 cells is essential for a T-dependent immune response. [68]

The ERK pathway is involved in the regulation of cell growth and differentiation. ERK1 and 2 are the 2 isoforms and are also called as p44/p42 MAP kinases. They can be activated by MEK1 and MEK2 upstream kinases. ERK activation is an important event of T cell activation. Involvement of TcR causes recruitment of molecular components to the cell surface, which in turn activate the sos-Ras-MEK-ERK pathway. Experiments also showed that the ERK pathway enhances IL-4 induced STAT-6 and IL-4 phosphorylation. This evidence suggests that there is a possibility of cross-regulation between the signaling pathways. [68]

The p38 kinase pathway was first activated in mouse Th1 effector cells. Studies also suggest that pro-inflammatory cytokines like IL-12 and IL-18 play a role in p38
activation in T cells. p38 kinases imidazole inhibitors block IFN-gamma production by Th1 cells in a dose-dependent manner but there is no effect on IL-4 production by Th2 cells. [68]

Three JNK genes have been identified in mammals: JNK 1, 2, 3. JNK 3 is present in neuronal tissues while JNK 1 and 2 are present almost everywhere. Northern blot analysis showed that JNK activity in naïve and activated T cells was very low for a short time (less than 30 minutes) but increased after T cell at about an hour. JNK 1 and 2 are expressed in equal amounts in Th1 and Th2 cells in the effector cells, but Th1 cells nevertheless have a high JNK activity. This shows that JNK may play an important role in Th1 cells.

Cytotoxic CD8+ T (Tc) cells are an important component of cellular immunity. Tc precursor cells proliferate with the help of IL-2 upon activation and produce IFN-gamma and differentiate into cytotoxic T cells. p38 regulates IFN-gamma production in CD8+ T cells as it does in CD4+ T cells. This could suggest that parallel pathways exist in CD4+ and CD8+ cells for IFN-gamma activation. [68]

4.7 Mitogen activated protein kinases and asthma:

Asthma is a chronic inflammatory disease of the airways. It is characterized by bronchoconstriction, T helper 2 (Th2) lymphocytes and mast cells, bronchial hyperresponsiveness and airway remodeling, inflammatory cell activation and recruitment, bronchial epithelium shedding and increased airway smooth muscle mass. These events are triggered by autacoids, cytokines, chemokines and growth factors. Mitogen activated protein kinases are an important part of the signaling pathways which are activated in inflammation, immunity, cell death and proliferation. These can have an
effect on asthma pathophysiology in Th2 cell differentiation, inflammatory cell infiltration and airway smooth muscle mass activity. [69-71] MAPK activation can be observed in immune inflammatory as well as bronchial cells. [72]

SAPK/JNK:

These are called stress-activated protein kinases and are triggered by cellular stresses and some pro-inflammatory cytokines. The MAPKKKs and MAPKKs are represented by MEKK1/2, and by JNK kinases MKK4/7, respectively. JNK substrates are c-Jun component of activator-protein 1 (AP-1), Elk-1 and ATF2, which after JNK-mediated activation plays an important role in stress responses, apoptosis and inflammation. [72]

Inflammatory cells:

A key pathogenic role is played by growth factors such as TGF-b, which promotes the transition from pulmonary fibroblasts to myofibroblasts through SAPK/JNK activation. This pathway is also used by the Th2 cytokines IL-4 and IL-13. SAPK/JNK also induces airway smooth muscle mass mitogenesis in rats. [72]

SAPK/JNK inhibitors:

Selective SAPK/JNK inhibitors are SP 600125 and SPC 105. They are quite effective in animal asthma models. In rats sensitive to allergens, SP 600125 reduced bronchial infiltration with eosinophils and T lymphocytes, as well as airway smooth muscle mass proliferation. SP 600125 was used in combination with SB 203580 and PD 98059, causing an inhibition in hydrogen peroxide-induced IL-8 secretion. [72]

ERK1/2:
Extracellular signal regulated kinases activation is involved in cell-cycle regulation and tissue proliferation. It can be induced by growth factors, cytokines and GPCRs. The phosphorylation cascade is initiated by the MAPKKK Raf-1, whose activation requires the interaction with a GTP-bound isoforms of the Ras protein family. ERK1/2 phosphorylates transcription factors such as STATs, Elk1, Ets1, Sap1a, cMyc and Tal. [72]

Inflammatory cells:

T helper lymphocytes are involved in asthmatic inflammation and they produce a Th2 cytokine pattern which includes IL-4, IL-5, IL-9, IL-13 and IL-25. The ERK1 pathway is required for Th2 differentiation. Activation of the Ras/ERK pathway subsequent to T cell receptor stimulation can direct the differentiation of naive T helper lymphocytes into the Th2 cells. IL-4 is capable of switching Th0 to Th2 phenotype, stimulating immunoglobulin E synthesis, and promotes eosinophil adhesion to vascular endothelium. IL-4 production and biological effects are mediated by the JAK (Janus kinases)-STAT (signal transducers and activators of transcription) family of cytokine receptor-associated tyrosine kinases. ERK1/2 may phosphorylate STATs thus stimulating their activity. [72]

ERK1/2 inhibitors:

The ERK1/2 pathway can be inhibited by a specific MEK1/2 inhibitor like PD 98059 and PD 184352. Incubation of eosinophils isolated from allergic donors with PD 98059 results in a decrease in chemotactic migration induced by eotaxin. PD 98059 also reduces release of leukotrienes from guinea pig lungs. [72]
p38 plays an important role in cellular responses to stress and pro-inflammatory stimuli by increasing the biosynthesis of cytokines and the recruitment of inflammatory cells. Activation of the p38 signal transduction pathway involves a specific MAPKKK like TGF-b-activated kinase 1 (TAK-1) and apoptosis signal-regulating kinase 1 (ASK-1), which phosphorylates the MAPKKs: M KK3 and M KK6, that are in turn responsible for p38 phosphorylation. p38 MAPK phosphorylates transcription factors like ATF2/6, Chop, Max, MEF2C, nuclear histones (H3) and heat shock proteins. [72]

Inflammatory cells:

Th2 secretory activity is affected by p38 which promotes the synthesis of IL-5 and IL-13. It is also capable of inhibiting eosinophil apoptosis. p38 MAPK can regulate mast cell migration toward antigen. It is also involved in neutrophil recruitment. p38 is also involved in mediating myofibroblast hyperplasia and collagen deposition caused by IL-6, whose production by lung fibroblasts is dependent on p38 activation. [72]

p38 inhibitors:

Several pyridinylimidazole inhibitors of p38 are available. The bicyclic pyridinylimidazole SKF 86002 and the 2,4,5-triaryl imidazole SB 203580 block the biosynthesis of pro-inflammatory cytokines. New strategies have identified a new class of highly potent p38 inhibitors, characterized by the replacement of the pyrazole ring with either an isoxazole or a thiophene. [72]
HYPOTHESIS

Histamine regulates the phosphorylation of ERK1/2 and SAPK/JNK.
SPECIFIC AIMS

ERK1/2:
- To demonstrate the effect of histamine on ERK1/2 phosphorylation.
- To identify the histamine receptors involved in ERK1/2 phosphorylation.
- To determine mechanism of ERK1/2 phosphorylation by histamine.

SAPK/JNK:
- To demonstrate the effect of histamine on SAPK/JNK phosphorylation.
- To identify the histamine receptors involved in SAPK/JNK phosphorylation.
- To determine mechanism of SAPK/JNK phosphorylation by histamine.
MATERIALS AND METHODS

Animals

C57/BL 6 mice, female, eight weeks old were purchased from Charles River, MA and TNF-alpha knockout C57/BL 6 mice, 4-8 weeks old were purchased from a live repository from Jackson Laboratories.

2.1 Materials

Phorbol 12 myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St.Louis, MO) and was used to activate the C57BL/6 splenocytes. The culture medium RPMI-1640, HEPES buffer, Hank’s balanced salt solution, sodium pyruvate, sodium bicarbonate, L-glutamine, 2-mercaptoethanol (2-ME), amphotericin B, 100 International units/ml penicillin and streptomycin, glucose were purchased from Sigma-Aldrich (St.Louis, MO). Histamine dihydrochloride, pyrilamine maleate, ranitidine dihydrochloride, betahistine dihydrochloride, amthamine dihydrobromide and thioperamide maleate were purchased from Sigma-Aldrich (St. Louis, MO). Methimepip dihydrobromide and 4-methylhistamine dihydrochloride were purchased from Tocris Biosciences (Ellisville, MO). The activated splenocytes were lysed with triton X-100 detergent from Biorad and trizma® base, ammonium chloride, potassium bicarbonate, sodium fluoride, sodium pyrophosphate, sodium orthovanadate, aprotinin bovine lung, ethylenediaminetetraaceticacid (EDTA), phenylmethylsulfonyl fluoride (PMSF), phosphatase inhibitor cocktail, protease inhibitor cocktail, fetal bovine serum (FBS), leupeptin hemisulfate salt and pepstatin A from Sigma-Aldrich (St.Louis, MO). Protein standard, laemmlli sample buffer, lyophilized bovine plasma gamma globulin, tween-20,
dye reagent concentrate, nitrocellulose membrane and blotting grade blocker non-fat dry milk were purchased from Bio-Rad laboratories (Hercules, CA). N, N, N’, N’-tetramethylethylenediamine (TEMED) and acrylamide were purchased from Sigma-Aldrich and glycine, methanol and sodium dodecyl sulfate were purchased from Fisher Scientific (Pittsburgh, PA) for gel electrophoresis. The phospho-MAPK family antibody sampler kit consisting of phospho-p44/42 MAPK (Erk1/2) rabbit mAb, phospho-p38 MAPK antibody, phospho-ERK1/2 rabbit mAb, U0126 (MEK1/2 Inhibitor), anti-rabbit IgG, HRP-linked antibody and the phospho-Erk1/2 pathway sampler kit consisting of phospho-c-Raf rabbit mAb, phospho-MEK1/2 rabbit mAb, phospho-p44/42 MAPK (Erk1/2) rabbit mAb, phospho-p90RSK antibody, phospho-MSK1 antibody, anti-rabbit IgG, HRP-linked antibody and U0126 (MEK1/2 inhibitor), biotinylated protein ladder, lumiglo reagent and peroxide solution were purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit IgG, HRP-linked antibody, anti-biotin, HRP-linked antibody and β-actin antibody were also purchased from Cell Signaling Technology (Beverly, MA). XAR films were purchased from Sigma Aldrich.

2.2 Isolation of Splenocytes

Under aseptic conditions, the 8 weeks old C57BL/6 splenocytes were removed from the mouse after cervical dislocation. The splenocytes were isolated under sterile conditions and were then suspended in cold Hank's balanced salt solution (HBSS, Sigma). The spleen was punctured using a plunger of a sterile needle and the splenocytes were flushed out in cold Hank's balanced salt solution. Splenocytes were homogenated and passed through a 0.7µm cell strainer into a 50 ml centrifuge tube. The volume was made up to 50 ml with cold HBSS and the tube was centrifuged at 1500 rpm for 10 minutes. The
supernatant was discarded and 5ml of lysis buffer I (KHCO3 1 mM, EDTA 0.01 mM, NH4Cl 150 mM) was used to suspend the cell pellet for 5 minutes in ice. The buffer action was stopped by resuspending the cells in 50 ml of cold HBSS and centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and the splenocytes were resuspended in RPMI complete medium (RPMI 1640, 1% HEPES buffer, 10% FBS, 50mM 2-ME, 2mM l-glutamate, 1% glucose, 2% sodium bicarbonate, 1% sodium pyruvate and antibiotics). Splenocytes were checked for >95% viability using trypan blue stain. The cell concentration was adjusted to 15-20 x 10^6 cells/ml in RPMI complete medium.

2.3 Treatment with Drugs

a) Effects of histamine and histamine receptor agonists on SAPK/JNK and ERK1/2 phosphorylation in C57BL/6 splenocytes:

The splenocytes (15-20 X 10^6/ml) were isolated and treated with histamine (10^-4 M – 10^-11 M), H1R agonist betaistine (10^-5 M) and histamine H2R agonist amthamine (10^-5 M), histamine H3R agonist methimepip dihydrobromide (10^-6 M) and histamine H4R agonist 4-methylhistamine dihydrochloride (10^-6 M) for 3 minutes for ERK1/2 and 30 minutes for SAPK/JNK. Phorbol 12 myristate 13-acetate (PMA) was used as a positive control. The treatment was stopped by adding lysis buffer I (KHCO3 1 mM, EDTA 0.01 mM, NH4Cl 150 mM) in ice for five minutes. The treated cells were centrifuged at 1500 rpm for 10 minutes to get a cell pellet. The cells were then lysed using freshly prepared lysis buffer II (2mM sodium orthovanadate, 10mM sodium fluoride, 1% triton-x-100, 10mM sodium pyrophosphate, pH 7.4, 1mM EDTA, 1mM PMSF, 50mM Tris, 10µg/ml of aprotinin bovine lung, 10µg/ml of pepstatin A, 10µg/ml of leupeptin hemisulfate salt,
10% protease inhibitor cocktail, 10% phosphatase inhibitor cocktail) and kept on dry ice for 45 minutes-1 hour. The cell lysate was then thawed at 4 degrees °Celsius and then centrifuged at 3000 rpm for 10 minutes.

2.4 Protein isolation and assay

The supernatants were collected and further assessed for the protein content. The concentration of protein for each cell lysate was determined using bovine plasma gamma globulin as a standard. Duplicate samples were made in Bradford’s protein assay dye and were assessed spectrophotometrically at a fixed wavelength of 592 nm. SDS PAGE loading buffer (1M Tris pH 6.6, glycine, 10% SDS, saturated bromophenol blue, ß-mercaptoethanol added fresh just before adding it to the samples) was used to prepare equal protein samples and then boiled for 5 minutes.

2.5 Western Blot Analysis

The samples were loaded in the wells and gel was run at 93 volts. 80µg protein was resolved by 10% SDS-PAGE in the running buffer (Tris, glycine and SDS). After about 1.5 hours, the gel was removed and separated from the plates.

The proteins were transferred to a nitrocellulose membrane overnight at 70 volts. The membrane was then removed and blocked with 5% non-fat dry milk blocking buffer for 1.5 hours at room temperature (RT). The membrane was washed three times for 5 minutes each with TBST (1X TBS, 0.01% Tween-20). The blocked membrane was then incubated with phospho-p44/42 MAPK (Erk1/2) rabbit monoclonal antibody (1:1000 each)/phospho-pSAPK/JNK MAPK rabbit monoclonal antibody overnight. The membrane was washed three times for 5 minutes each with TBST (1X TBS, 0.01%
Tween-20). The membrane was then incubated with HRP-conjugated secondary antibodies (1:2000) in 10ml of TBST for 1 hour at room temperature after which the membrane was washed three times for 5 minutes each with TBST. The protein bands on the membrane were visualized by enhanced chemiluminescence using the lumiglo and peroxide solution and phosphorylated ERK1/2 and SAPK/JNK was analyzed by Western Blot Analysis. The same membranes were stripped to analyze the total basal ERK1/2 and SAPK/JNK levels using the stripping buffer (2% SDS, 62.5M Tris, 100mM 2- ME) for 30 minutes at 50°C. The membrane was washed twice for 5 minutes with TBST and again blocked with 5% non-fat dry milk for 1.5 hours. The membrane was washed once with TBST and reprobed with beta-actin antibody to determine the basal ERK1/2 levels.

2.6 Statistical analysis

The quantification of the density of bands was done by densitometry and the statistical analysis was performed by one-way analysis of variance (ANOVA). The data is representative of three experiments. *p<0.05 (n=3) and **p<0.01 (n=3) [* * is the statistical significance between control and the treatment groups].

b) Effects of histamine receptor antagonists on ERK1/2 and SAPK/JNK phosphorylation in C57BL/6 splenocytes:

Splenocytes (15-20 X 10^6/ml) were pretreated with H1R antagonist pyrilamine (10^{-6} M), H2R antagonist ranitidine (10^{-5} M), H3/H4R antagonist thioperamide (10^{-6} M) and H3R antagonist clobenpropit (10^{-5} M) followed by treatment with histamine (10^{-4} M) for 3 minutes for ERK1/2 and 30 minutes for SAPK/JNK. Phorbol 12 myristate 13-acetate (PMA) was used as a positive control. The treated cells were then lysed and phosphorylated ERK1/2 and SAPK/JNK levels were analyzed by western blot analysis as
described above. The quantification of the density of bands was done by densitometry
and the statistical analysis was performed by one-way analysis of variance (ANOVA).
The data is representative of three experiments. *p<0.05 (n=3) and **p<0.01 (n=3) {*, **
is the statistical significance between control and the treatment groups}. ^p<0.05 (n=3)
and ^^p<0.01 (n=3) {^, ^^ is the statistical significance between histamine alone and the
antagonists pretreated with histamine}.

c] Effects of histamine on phosphorylation of ERK2 and SAPK/JNK in TNF-alpha
knockout mice:

The splenocytes (15-20 X 10^6/ml) from TNF-alpha knockout mice were isolated. They
were treated with histamine (10^-4 M), histamine H1R agonist betaistine (10^-5 M),
histamine H2R agonist amthamine (10^-5 M), histamine H3R agonist methimepip
dihydrobromide (10^-6 M) and histamine H4R agonist 4-methylhistamine dihydrochloride
for 3 minutes for ERK1/2 and 30 minutes for SAPK/JNK. Phorbol 12 myristate 13-
acetate (PMA) was used as a positive control. The treated cells were then lysed and
phosphorylated ERK1/2 and SAPK/JNK levels were analyzed by western blot analysis as
described previously. The quantification of the density of bands was done by
densitometry and the statistical analysis was performed by one-way analysis of variance
(ANOVA). The data is representative of three experiments. *p<0.05 (n=3) and **p<0.01
(n=3) {*, ** is the statistical significance between control and the treatment groups}. 
RESULTS

A) ERK1/2:

3.1] Effects of histamine on phosphorylation of ERK1/2 in C57BL/6 splenocytes:

Kinetic studies were performed to determine the optimum incubation time required for the phosphorylation of ERK2 using PMA (phorbol ester phorbol 12-myristate 13-acetate:10ng/ml) as a positive control. The levels of phosphorylation of ERK2 in response to \(10^{-4}\) M histamine were determined by western blot analysis. The control band showed constitutive activity. Optimum phosphorylation of ERK2 was observed at 3 minutes (Figure 3.1a). There was no effect of histamine on ERK1 phosphorylation. The splenocytes were then treated with different concentrations of histamine \(10^{-11}\) M and phosphorylation of ERK2 was assessed. Figure 2 shows effects of histamine \(10^{-4}\) M - \(10^{-8}\) M) on C57BL/6 splenocytes at 37 °C, 5% CO₂. There was an increase in ERK2 phosphorylation by histamine at \(10^{-4}\) M. At other concentrations, \(10^{-6}\) M- \(10^{-8}\) M, \(10^{-9}\) M - \(10^{-11}\) M, histamine did not significantly affect the phosphorylation of ERK2.
Figure 3.1a: Effects of kinetics of histamine on the phosphorylation of ERK1/2.

C57BL/6 splenocytes were treated with histamine ($10^{-4}$ M) for 1 minute, 3 minutes, 5 minutes and 15 minutes using PMA as a positive control. The cells were then lysed and ERK1/2 levels were determined by western blot analysis. a) Lane 1: untreated; lane 2: PMA; lane 3: 1 minute ; lane 4: 3 minutes ; Lane 5: 5 minutes ; Lane 6: 15 minutes b) The bar graph shows the ratio of phosphorylated ERK1/2 to total non phosphorylated ERK1/2 based on the densitometry of immunoblots; the average of 3 experiments was taken.
Figure 3.2 a: Effects of high concentrations of histamine on the phosphorylation of ERK1/2. C57BL/6 splenocytes were treated with histamine ($10^{-4}$ M to $10^{-8}$ M) for 3 minutes using PMA as a positive control. The cells were then lysed and ERK1/2 levels were determined by western blot analysis. a) Lane 1: untreated; lane 2: PMA; lane 3: histamine $10^{-4}$ M; lane 4: histamine $10^{-6}$ M; Lane 5: histamine $10^{-8}$ M b) The bar graph shows the ratio of phosphorylated ERK1/2 to total non-phosphorylated ERK1/2 based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
Figure 3.3a: Effects of low concentrations of histamine on the phosphorylation of ERK1/2.

C57BL/6 splenocytes were treated with histamine (10^{-9} M to 10^{-11} M) for 3 minutes using PMA as a positive control. The cells were then lysed and ERK1/2 levels were determined by western blot analysis. a) Lane 1: untreated; Lane 2: PMA; Lane 3: histamine 10^{-9} M; Lane 4: histamine 10^{-10} M; Lane 5: histamine 10^{-11} M b) The bar graph shows the ratio of phosphorylated ERK1/2 to total non phosphorylated ERK1/2 based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.4a] Effects of selective H1 and H2 agonists on ERK1/2 phosphorylation in C57BL/6 splenocytes:

To study the effects of selective histamine receptor agonists, C57BL/6 mice splenocytes (15-20 X 10^6/ml) were treated with H1 agonist betahistine (10^{-5}M) and H2 agonist amthamine (10^{-5}M) for 3 minutes at 37°C, 5% CO_2. PMA (phorbol ester phorbol 12-myristate 13-acetate: 10ng/ml) was used as a positive control. The cells were then lysed and the phosphorylated ERK2 was analyzed by western blot analysis. The control band showed constitutive activity. There was increase in the phosphorylation of ERK2 in the presence of betahistine (10^{-5} M) and amthamine (10^{-5} M) (Figure 3.4a). H1 and H2 agonists, however did not affect ERK1 phosphorylation. (Data not shown) These observations suggested that both H1 and H2 receptors were involved in the phosphorylation of ERK2.
Figure 3.4a: Effects of histamine receptor H1 and H2 agonists on phosphorylation of ERK1/2.

C57BL/6 splenocytes were treated with H1 receptor agonist betahistine (10^{-5} M) and H2 receptor agonist amthamine (10^{-5} M) for 3 minutes using PMA as a positive control. The cells were then lysed and the phosphorylated ERK1/2 was determined using the western blot analysis. a) lane 1: Untreated; lane 2: PMA lane 3: histamine 10^{-4} M; Lane 4: betahistine 10^{-5} M; Lane 5: amthamine 10^{-5} M. b) The bar graph shows the ratio of phosphorylated ERK1/2 to total non phosphorylated ERK1/2 based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.5a] Effects of selective H3 and H4 agonists on ERK1/2 phosphorylation in C57BL/6 splenocytes:

To study the effects of H3 and H4 receptor agonists, C57BL/6 mice splenocytes (15-20 X 10^6/ml) were treated with H3 agonist methimepip (10^-6 M) and H4 agonist 4-methyl histamine (10^-6 M) at 37°C, 5% CO2. PMA (phorbol ester phorbol 12-myristate 13-acetate 10ng/ml) was used as a positive control. The cells were then lysed and the phosphorylated ERK2 was analyzed by western blot analysis. The control band showed constitutive activity. As shown in figure 3.5a, both methimepip (10^-6 M) and 4-methyl histamine (10^-5 M) increased the phosphorylation of ERK2 suggesting that H3 and H4 receptors were also involved in ERK2 phosphorylation. There was no effect on ERK1 phosphorylation by either H3 or H4 agonists. (Data not shown)
Figure 3.5a: Effects of histamine receptor H3 and H4 agonists on phosphorylation of ERK1/2.

C57BL/6 splenocytes were treated with H3 receptor agonist methimepip (10^{-6} M) and H4 agonist 4-methyl histamine (10^{-6} M), for 3 minutes using PMA as a positive control. The cells were then lysed and the phosphorylated ERK1/2 was determined using western blot analysis. a) Lane 1: Untreated; lane 2: PMA; Lane 3: histamine 10^{-4} M; Lane 4: methimepip 10^{-6} M; Lane 5: 4-methyl histamine 10^{-6} M. b) The bar graph shows the ratio of phosphorylated ERK1/2 to total non phosphorylated ERK1/2 based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.6a] Effects of selective H1 and H2 antagonists on ERK1/2 phosphorylation in C57BL/6 splenocytes:

It was surprising to observe that all four histamine receptor subtypes were involved in ERK2 phosphorylation. To further confirm these results obtained with various histamine receptor agonists, histamine receptor antagonists were used. The C57BL/6 mice splenocytes (15–20 X 10^6/ml) were pretreated with H1R antagonist pyrilamine (10^{-6} M), H2R antagonist ranitidine (10^{-5} M) followed by treatment with histamine (10^{-4} M) at 37^oC, 5% CO₂. PMA (phorbol ester phorbol 12-myristate 13-acetate10ng/ml) was used as a positive control. The cells were then lysed and the phosphorylated ERK2 was analyzed by western blot analysis. The control band showed constitutive activity. As shown in Figure 3.6a, pyrilamine (10^{-6} M), and ranitidine (10^{-5} M), inhibited the effects of histamine (10^{-4} M) on ERK2 phosphorylation. H1 and H2 antagonists, treated with histamine did not show any effect on ERK1 phosphorylation. (Data not shown)
Figure 3.6a: Effects of histamine receptors H1 and H2 antagonists on ERK1/2 phosphorylation.

C57BL/6 splenocytes were pretreated with H1 receptor antagonist pyrilamine (10^{-6} M) and H2 receptor antagonist ranitidine (10^{-5} M) followed by treatment with histamine (10^{-4} M), for 3 minutes using PMA as a positive control. The cells were then lysed and the phosphorylated ERK1/2 was determined using the western blot analysis. a) Lane 1: Untreated; lane 2: PMA; Lane 3: histamine 10^{-4} M; Lane 4: histamine 10^{-4} M + ranitidine 10^{-5} M; Lane 5: histamine 10^{-4} M + pyrilamine 10^{-6} M. b) The bar graph shows the ratio of phosphorylated ERK1/2 to total non phosphorylated ERK1/2 based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.7a] Effects of specific H3/H4 receptor antagonists on histamine induced ERK1/2 phosphorylation in C57BL/6 splenocytes:

The C57BL/6 mice splenocytes (15–20 X 10⁶/ml) were pretreated with H3/H4 receptor antagonist thioperamide (10⁻⁶ M) and an H3 receptor antagonist, clobenpropit (10⁻⁵ M), followed by treatment with histamine (10⁻⁴ M) at 37°C, 5% CO₂. PMA (phorbol ester phorbol 12-myristate 13-acetate) was used as a positive control. The cells were then lysed and the phosphorylated ERK2 was analyzed by western blot analysis. The control band showed constitutive activity. As shown in figure 3.7a, thioperamide (10⁻⁶ M), and clobenpropit (10⁻⁵ M), inhibited the effects of histamine (10⁻⁴ M) on ERK2 phosphorylation. This further confirmed the data obtained with H3 and H4 receptor agonists. H3 and H4 antagonists treated with histamine did not show any effect on ERK1 phosphorylation. (Data not shown)
Figure 3.7a: Effects of histamine receptors H3 and H4 antagonists on ERK1/2 phosphorylation.

C57BL/6 splenocytes were pretreated with H3/H4 receptor antagonist thioperamide ($10^{-6}$ M) and a potent H3 antagonist, clobenpropit ($10^{-5}$ M), followed by treatment with histamine ($10^{-4}$ M), for 3 minutes using PMA as a positive control. The cells were then lysed and the phosphorylated ERK1/2 was determined using the western blot analysis. a) Lane 1: Untreated; lane 2: PMA; Lane 3: histamine $10^{-4}$ M; Lane 4: histamine $10^{-4}$ M + thioperamide $10^{-6}$ M; Lane 5: histamine $10^{-4}$ M + clobenpropit $10^{-5}$ M b) The bar graph shows the ratio of phosphorylated ERK1/2 to total non phosphorylated ERK1/2 based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.8a] Effects of histamine on ERK1/2 phosphorylation in TNF-alpha -/- mice:

Effects of histamine on ERK1/2 phosphorylation were assessed by using TNF-alpha knockout mice. Splenocytes (C57BL/6 TNF-alpha^−/−) (15–20 X 10^6/ml) were treated with histamine (10^-4 M), specific H1 receptor agonist betahistine (10^-5 M), H2 receptor agonist amthamine (10^-5 M), H3 receptor agonist methimepip (10^-6 M) and H4 receptor agonist 4-methyl histamine (10^-6 M) for 3 minutes. PMA (phorbol ester phorbol 12-myristate 13-acetate: 10ng/ml) was used as a positive control for this experiment. The cells were then lysed and the phosphorylated ERK2 was analyzed by western blot analysis. The control band showed constitutive activity. As shown in Figure 3.8a, there was inhibition of ERK1 and ERK2 phosphorylation by H2, H3 and H4 agonists. The effect on ERK2 phosphorylation by H1 agonist was not significant.
Figure 3.8a: Effect of histamine on phosphorylation of ERK1/2 in TNF-alpha / mice splenocytes.

C57BL/6 splenocytes were treated with specific H1 receptor agonist betahistine (10^{-5} M), H2 receptor agonist amthamine (10^{-5} M), H3 receptor agonist methimepip (10^{-6} M), H4 receptor agonist 4-methyl histamine (10^{-6} M) and histamine (10^{-4} M) for 3 minutes using PMA as a positive control. The cells were then lysed and the phosphorylated ERK1/2 was analyzed by western blot analysis. a) Lane 1: Untreated; lane 2: PMA; Lane 3: histamine 10^{-4} M; Lane 4: betaistine 10^{-5} M; Lane 5: amthamine 10^{-5} M; Lane 6: methimepip 10^{-6} M; Lane 7: 4-methyl histamine 10^{-6} M. The bar graph shows the ratio of phosphorylated ERK1/2 to total non phosphorylated ERK1/2 based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
B] SAPK/JNK:

3.1] Effects of histamine on phosphorylation of SAPK/JNK in C57BL/6 splenocytes:

Kinetic studies were performed to determine the optimum incubation time required for the phosphorylation of SAPK/JNK by histamine using PMA (phorbol ester phorbol 12-myristate 13-acetate: 10ng/ml) as a positive control. The levels of phosphorylation of SAPK/JNK were determined by western blot analysis. The control band showed constitutive activity. SAPK/JNK at 46kDa was affected. Optimum phosphorylation of SAPK/JNK was observed at 30 minutes (data not shown). Figure 3.1b shows phosphorylation of SAPK/JNK after C57BL/6 splenocytes were treated with different concentrations of histamine ($10^{-4}$ M-$10^{-8}$ M) at 37 degrees C, 5% CO$_2$. Histamine ($10^{-4}$ M-$10^{-8}$ M) showed a decrease in phosphorylation of SAPK/JNK at these concentrations, but the optimum decrease was seen at ($10^{-4}$ M). Histamine did not show any significant effect on phosphorylation of SAPK/JNK at lower concentrations ($10^{-9}$ M -$10^{-11}$ M) (Figure 3.2b)
Figure 3.1b: Effects of histamine on the phosphorylation of SAPK/JNK.

C57BL/6 splenocytes were treated with histamine ($10^{-4}$ M to $10^{-8}$ M) for 30 minutes using PMA as a positive control. The cells were then lysed and SAPK/JNK levels were determined by western blot analysis. a) Lane 1: untreated; lane 2: PMA; lane 3: Histamine $10^{-4}$ M; lane 4: Histamine $10^{-6}$ M; Lane 6: Histamine $10^{-8}$ M b) The bar graph shows the ratio of phosphorylated SAPK/JNK to total non phosphorylated SAPK/JNK based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
Figure 3.2b: Effects of low concentrations of histamine on the phosphorylation of SAPK/JNK.

C57BL/6 splenocytes were treated with histamine ($10^{-9}$ M to $10^{-11}$ M) for 30 minutes using PMA as a positive control. The cells were then lysed and SAPK/JNK levels were determined by western blot analysis. a) Lane 1: untreated; Lane 2: PMA; lane 3: Histamine $10^{-9}$ M; lane 4: Histamine $10^{-10}$ M; lane 5: Histamine $10^{-11}$ M b) The bar graph shows the ratio of phosphorylated SAPK/JNK to total non phosphorylated SAPK/JNK based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.3b] Effects of selective H1 and H2 agonists on SAPK/JNK phosphorylation in C57BL/6 splenocytes:

To study the effects of specific histamine receptor agonists, C57BL/6 mice splenocytes (15-20 X 10^6/ml) were treated with H1R agonist betahistine (10^{-5}M) and H2R agonist amthamine (10^{-5}M) for 30 minutes using PMA (phorbol ester phorbol 12-myristate 13 acetate: 10ng/ml) as a positive control at 37 degrees C, 5% CO₂. The cells were then lysed and the phosphorylated SAPK/JNK was analyzed by western blot analysis. The control band showed constitutive activity. There was decrease in the phosphorylation of SAPK/JNK (46kDA) in the presence of betahistine (10^{-5} M) (Figure 3.3b) and amthamine (10^{-5} M) did not show any effect (Figure 3.4b, 46kDA). The observations suggested that the decrease in SAPK/JNK phosphorylation by histamine was mediated via H1R.
Figure 3.3b: Effects of histamine receptor H1 agonists on phosphorylation of SAPK/JNK
C57BL/6 splenocytes were treated with H1 receptor agonist betahistine ($10^{-5}$ M) for 30
minutes using PMA as a positive control. The cells were then lysed and the
phosphorylated SAPK/JNK was determined using western blot analysis. a) lane 1:
Untreated; lane 2: PMA lane 3: Histamine $10^{-4}$ M; lane 4: Betahistine $10^{-5}$ M; b) The bar
graph shows the ratio of phosphorylated SAPK/JNK to total non phosphorylated
SAPK/JNK based on the densitometry of immunoblots. The band density ratio was
quantified by densitometry and analyzed by single factor analysis of variance (ANOVA);
the average of 3 experiments was taken.
Figure 3.4b: Effects of histamine receptor H2 agonists on phosphorylation of SAPK/JNK. C57BL/6 splenocytes were treated with H1 receptor agonist amthamine ($10^{-5}$ M) for 30 minutes using PMA as a positive control. The cells were then lysed and the phosphorylated SAPK/JNK was determined using western blot analysis. a) Lane 1: Untreated; lane 2: PMA; Lane 3: Histamine $10^{-4}$ M; Lane 4: Amthamine $10^{-5}$ M. b) The bar graph shows the ratio of phosphorylated SAPK/JNK to total non phosphorylated SAPK/JNK based on the densitometry of immunoblot. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.5b] Effects of selective H3 and H4 agonists on SAPK/JNK phosphorylation in C57BL/6 splenocytes:

To study the effects of H3 and H4 agonists, C57BL/6 mice splenocytes (15-20 X 10^6/ml) were treated with H3R agonist methimepip (10^{-6}M) and H4R agonist 4-methyl histamine (10^{-6}M) using PMA (phorbol ester phorbol 12-myristate 13-acetate 10ng/ml) as a positive control at 37 degrees C, 5% CO_2. The cells were then lysed and the phosphorylated SAPK/JNK was analyzed by western blot analysis. The control band showed constitutive activity. As shown in figure 3.5b, both methimepip (10^{-6}M) and 4-methyl histamine (10^{-6}M) caused an increase in the phosphorylation of SAPK/JNK (46kDA) suggesting a role of H3 and H4 receptors in SAPK/JNK phosphorylation.
Figure 3.5b: Effects of histamine receptor H3 and H4 agonists on phosphorylation of SAPK/JNK.

C57BL/6 splenocytes were treated with H3 receptor agonist methimepip (10^{-6} M) and H4 agonist 4-methyl histamine (10^{-6} M) for 30 minutes using PMA as a positive control. The cells were then lysed and the phosphorylated SAPK/JNK was determined using western blot analysis. a) Lane 1: Untreated; lane 2: PMA; Lane 3: Histamine 10^{-4} M; Lane 4: Methimepip 10^{-6} M; Lane 5: 4-Methyl Histamine 10^{-6} M. b) The bar graph shows the ratio of phosphorylated SAPK/JNK to total non phosphorylated SAPK/JNK based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.6b] Effects of selective H1 and H2 receptor antagonists on SAPK/JNK phosphorylation in C57BL/6 splenocytes:

The C57BL/6 mice splenocytes (15–20 X 10^6/ml) were pretreated with H1 receptor antagonist pyrilamine (10^-6 M), H2 receptor antagonist ranitidine (10^-5 M) followed by treatment with histamine (10^-4 M) using PMA (phorbol ester phorbol 12-myristate 13 acetate: 10ng/ml) as a positive control at 37 degrees C, 5% CO₂. The cells were then lysed and the phosphorylated SAPK/JNK was analyzed by western blot analysis. The control band showed constitutive activity. As shown in Figure 3.6b, pyrilamine (10^-6 M), and ranitidine (10^-5 M), partially reversed the effects of histamine (10^-4 M) on the inhibition of SAPK/JNK phosphorylation. (46kDA)
Figure 3.6b: Effects of histamine receptors H1 and H2 antagonists on SAPK/JNK phosphorylation.

C57BL/6 splenocytes were pretreated with H1 receptor antagonist pyrilamine (10^{-6} M) and H2 receptor antagonist ranitidine (10^{-5} M) followed by treatment with histamine (10^{-4} M), for 30 minutes using PMA as a positive control. The cells were then lysed and phosphorylated SAPK/JNK was determined using western blot analysis. a) Lane 1: Untreated; lane 2: PMA; Lane 3: Histamine 10^{-4} M; Lane 4: Histamine 10^{-4} M + pyrilamine 10^{-6} M; Lane 5: Histamine 10^{-4} M + Ranitidine 10^{-5} M. b) The bar graph shows the ratio of phosphorylated SAPK/JNK to total non phosphorylated SAPK/JNK based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.7b] Effects of specific H3/H4 receptor antagonists on histamine induced 
PAPK/JNK phosphorylation in C57BL/6 splenocytes:

The C57BL/6 mice splenocytes (15–20 X 10^6/ml) were pretreated with H3/H4 R 
antagonist thioperamide (10^-6 M) and a H3R antagonist, clobenpropit (10^-5 M), followed 
by treatment with histamine (10^-4 M) using PMA (phorbol ester phorbol 12-myristate 13 
acetate) as a positive control at 37 degrees C, 5% CO₂. The cells were then lysed and the 
phosphorylated SAPK/JNK was analyzed by western blot analysis. The control band 
showed constitutive activity. As shown in figure 3.7b, thioperamide (10^-6 M), and 
clobenpropit (10^-5 M), partially reversed the inhibitory effects of histamine (10^-4 M) on 
PAPK/JNK phosphorylation, although H3 and H4 agonists did not inhibit SAPK/JNK 
phosphorylation.
Figure 3.7b: Effects of histamine receptors H3 and H4 antagonists on SAPK/JNK phosphorylation.

C57BL/6 splenocytes were pretreated with H3/H4 receptor antagonist thioperamide (10\(^{-6}\)M) and a potent H3 antagonist, clobenpropit (10\(^{-5}\) M), followed by treatment with histamine (10\(^{-4}\) M), for 30 minutes using PMA as a positive control. The cells were then lysed and the phosphorylated SAPK/JNK was determined using western blot analysis. a) Lane 1: Untreated; lane 2: PMA; Lane 3: Histamine 10\(^{-4}\) M; Lane 4: Histamine 10\(^{-4}\) M + Thioperamide 10\(^{-6}\) M; Lane 5: Histamine 10\(^{-4}\) M + Clobenpropit 10\(^{-5}\) M. b) The bar graph shows the ratio of phosphorylated SAPK/JNK to total non phosphorylated SAPK/JNK based on the densitometry of immunoblots. The band density ratio was quantified by
densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
3.8b] Effects of histamine on SAPK/JNK phosphorylation in TNF-alpha -/- mice:

Effects of histamine on SAPK/JNK phosphorylation were assessed by using TNF-alpha knockout mice. Splenocytes (C57BL/6 TNF-alpha−/ −) (15–20 X 10^6/ml) were treated with histamine (10^{-4} M), specific H1R agonist betahistine (10^{-5} M), H2R agonist amthamine (10^{-5} M), H3 receptor agonist methimepip (10^{-6} M) and H4R agonist 4-methyl histamine (10^{-6} M) for 30 minutes using PMA (phorbol ester phorbol 12-myristate 13 acetate: 10ng/ml) as a positive control at 37 degrees C, 5% CO_2. The cells were then lysed and the phosphorylated SAPK/JNK was analyzed by western blot analysis. The control band showed constitutive activity. As shown in Figure 3.8b, histamine (10^{-4} M) inhibited SAPK/JNK phosphorylation (46kDA) in the absence of TNF-alpha. H1 receptor agonist betahistine (10^{-5} M), H2 receptor agonist amthamine (10^{-5} M), H3 receptor agonist methimepip (10^{-6} M), inhibited the phosphorylation of SAPK/JNK. (46kDA) H4 agonist did not exhibit any effect on the phosphorylation of SAPK/JNK (46kDA) in TNF-alpha knockout splenocytes.
Figure 3.8b: Effects of histamine on phosphorylation of SAPK/JNK in TNF-alpha^-/- mice splenocytes.

C57BL/6 splenocytes were treated with specific H1 receptor agonist betahistine (10^-5 M), H2 receptor agonist amthamine (10^-5 M), H3 receptor agonist methimepip (10^-6 M), H4 receptor agonist 4-methyl histamine (10^-6 M) and histamine (10^-4 M) for 30 minutes using PMA as a positive control. The cells were then lysed and the phosphorylated SAPK/JNK was analyzed by western blot analysis. a) Lane 1: Untreated; lane 2: PMA; Lane 3: Histamine 10^-4 M; Lane 4: Betahistine 10^-5 M ; Lane 5: Amthamine 10^-5 M ; Lane 6: Methimepip 10^-6 M; Lane 7: 4-Methyl histamine 10^-6 M; b) The bar graph shows the ratio of phosphorylated SAPK/JNK to total non phosphorylated SAPK/JNK based on the densitometry of immunoblots. The band density ratio was quantified by densitometry and analyzed by single factor analysis of variance (ANOVA); the average of 3 experiments was taken.
DISCUSSION

This study was designed to evaluate the effects of histamine on two mitogen activated protein kinases: ERK1/2 and SAPK/JNK phosphorylation.

Kinetic studies were performed and ERK1/2 was optimally phosphorylated at 3 minutes. Histamine \(10^{-4}\) M upregulated the phosphorylation of ERK2. Localized concentrations of histamine as high as \(10^{-4}\) M have been reported in specific inflammatory responses. [73] There was no effect on ERK2 phosphorylation at other concentrations of histamine \(10^{-6}\) M - \(10^{-11}\) M). Furthermore, there was no effect of histamine on ERK1 phosphorylation suggesting that these effects on ERK2 phosphorylation were selective.

Histamine affects the balance of T helper type 1 (Th1) and T helper type 2 (Th2) cytokines by shifting the balance from a Th1 to a Th2 pattern. [74] Histamine is involved in allergic diseases like asthma by increasing the secretion of Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13 and by decreasing the secretion of Th1 cytokines IL-2 and IFN-gamma and the monokine IL-12. [74] The control band showed constitutive activity. The constitutive receptor is capable of producing its own biological response in the absence of a bound ligand. [75] Specific histamine receptor subtypes involved were determined. H1 agonist betahistine \(10^{-5}\) M augmented the phosphorylation of ERK2. This was further proved by using H1 antagonist pyrilamine \(10^{-6}\) M which inhibited the effects of histamine on the phosphorylation of ERK2. Cammarota et al [76] investigated the role of ERK1/2 in the histamine induced tyrosine hydroxylase phosphorylation in bovine adrenal chromaffin cells. They found that there was an increase in the phosphorylation of both ERK1 and ERK2 which was mediated by H1 receptors. They reported that ERK1/2 up regulation caused by histamine was involved in tyrosine hydroxylase activation. [76]
H2 agonist amthamine (10^{-5} M) also augmented the phosphorylation of ERK2. H2 receptors associate with G_{alpha} and increase cAMP production which induces ERK1/2 phosphorylation. [77] It has been reported that agents which increased cAMP were involved in ERK1/2 phosphorylation. Lee and Esselman have shown that increase in intracellular cAMP with forskolin resulted in ERK1/2 phosphorylation in jurkat T-lymphocytes. [77] In our study, H3 agonist methimepip (10^{-6} M) and H4 agonist 4-methyl histamine (10^{-6} M) also augmented the phosphorylation of ERK2. H3/H4 antagonist thioperamide (10^{-6} M) and clobenpropit (10^{-5} M) reversed the effect of histamine on ERK2 phosphorylation.

H3 receptors inhibit cAMP formation and increase calcium mobilization. Francis et al showed the involvement of H3 receptor agonist in inhibiting biliary growth of rats by down regulating the cAMP dependent ERK1/2 pathway. [78] This contrasted with our results, which showed that there was an increase in ERK2 phosphorylation by an H3 agonist. Levi et al showed that H3 receptor mediated the decrease of norepinephrine exocytosis in cardiac sympathetic nerves. They reported that this decrease is via MAPK activation. H3 agonist imetit activates MAPK and when specific ERK inhibitors are used, there was a decrease in the ant-exocytotic effect of imetit. They showed that there was ERK1/2 activation in the H3 receptor mediated attenuation of NE exocytosis. [79]

H4 receptors inhibit cAMP formation and increase intracellular calcium. In our study, there was an increase in ERK2 phosphorylation by an H4 agonist. This was compared to observations made by Gutzmer et al who found that there was phosphorylation of ERK1/2 by histamine in human monocyte-derived dendritic cells. They determined the involvement of ERK1/2 by using a MEK inhibitor, which blocked ERK1/2
phosphorylation and also reversed the H4 receptor mediated IL-12p70 suppression. [80, 81] Morse et al showed that human embryonic kidney -293 cells transfected with H4 receptors showed an intracellular calcium increase, but did not show histamine mediated cAMP inhibition, which is a characteristic of H4 receptors. However, they reported that there was an increase in mitogen activated protein kinase phosphorylation by histamine via H4 receptors. [82] Figure 7 shows the diagrammatic representation of ERK2 data in wild type mice.

Eosinophil degranulation and eotaxin chemotaxis which are involved in asthmatic inflammations activate ERK2. [72] Proinflammatory cytokines such as TNF-alpha play an important role in asthma. There is an increased level of TNF-alpha in asthmatic airways. Mast cells, macrophages, eosinophils and epithelial cells generate TNF-alpha, which is released during an allergen challenge. TNF-alpha increases the production of IL-8, GM-CSF (granulocyte macrophage colony stimulating factor) and RANTES (regulated
on activation, normal T expressed and secreted) by airway epithelial cells and also increases the adhesion molecule expression including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), whose involvement in the inflammatory cell recruitment is well known. TNF-alpha is also involved in tissue remodeling and increases myofibroblasts proliferation and fibroblast mitogenicity. [83] TNF-alpha also causes phosphorylation of ERK1/2. [84, 85] TNF-alpha knockout mice were used to determine whether histamine and its receptors acted via secretion of TNF-alpha to affect ERK2 phosphorylation.

We found that histamine (10^{-4} M) inhibited ERK2 phosphorylation in TNF-alpha knockout splenocytes and this inhibition was mediated by histamine H2, H3 and H4 receptors. H1 agonist betahistine, on the other hand, did not affect the phosphorylation of ERK2 in TNF-alpha knockout mice, while there was no effect on ERK1 phosphorylation by histamine in wild type mice. ERK1 phosphorylation was inhibited in TNF-alpha knockout mice by histamine H2, H3 and H4 receptors. These results suggest that dephosphorylation of ERK1/2 in the absence of TNF-alpha involved additional mechanisms. It has been suggested that IL-22, a relative of the IL-10 family, may be involved in ERK1/2 dephosphorylation. It influences a number of immune reactions and has an inhibitory effect on the signaling pathways which promote cell proliferation. Weber et al [86] showed that exposure of EMT 6 murine breast cancer cells to IL-22 resulted in decreased tumor growth by inhibition in ERK1/2 levels. [86] Another cytokine, SOCS-3 (suppressor of cytokine signaling-3) may also be involved in ERK1/2 dephosphorylation. Woolson et al [87] reported that there was a decrease in ERK1 and ERK2 activity by cyclic AMP via SOCS-3 in human umbilical vein endothelial cells.
(HUVECs) It is known that histamine H2 receptors are $G_s$ alpha proteins activate the cAMP pathway by activating adenylate cyclase. It could be speculated that H2 receptors are playing a role in the SOCS-3 inhibition of ERK1/2 via activation of cAMP pathway. Figure 8 below depicts the involvement of histamine and its receptors via other mechanisms on ERK2 phosphorylation.

Weidinger et al [88] showed that protease activated receptor-2 activation by the mast cell product tryptase via ERK1/2 phosphorylation inhibited sperm motility. [88] Ancha et al [89] showed that there was a dose dependent increase by histamine of matrix metalloproteinase in gastric epithelial cells. This was associated with ERK1/2 activation. When ERK1/2 was inhibited, there was inhibition of histamine induced metalloproteinase secretion too. [89] Cammarota et al [76] investigated the role of ERK1/2 in the histamine induced tyrosine hydroxylase phosphorylation in bovine adrenal chromaffin cells. They
found that there was an increase in the phosphorylation ERK1/2. [76] Kanda and Watanabe [90] investigated the effects of histamine on nerve growth factor production in keratinocytes. They found that histamine induced nerve growth factor activation caused c-fos expression, the phosphorylation of ERK1/2, which was inhibited by pyrilamine and PD98059, which is an inhibitor of MAPKK1. [90]

In summary, H1, H2, H3 and H4 receptors augmented ERK2 phosphorylation in C57BL/6 mice. However, in TNF-alpha knockout mice, H2, H3 and H4 agonists caused dephosphorylation of ERK2. Wild type and knock out data are shown in the diagrammatic representation below. It is interesting to note the involvement of H3 and H4 receptors in the secretion of TNF-alpha. The study provides evidence that H4 antagonists may be clinically relevant in treating asthmatic inflammation which is difficult to treat with the exception of glucocorticoids.

<table>
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<tr>
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<th>Histamine</th>
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Similar experiments were performed with SAPK/JNK in C57BL/6 splenocytes too.

Kinetic studies were done to determine the optimum time for SAPK/JNK phosphorylation. SAPK/JNK was optimally phosphorylated at 30 minutes. Histamine \((10^{-4} \text{ M}-10^{-8} \text{ M})\) inhibited the phosphorylation of SAPK/JNK. Local concentrations of histamine as high as \((10^{-4}\text{ M})\) have been reported in specific inflammatory responses [73]. Histamine did not show any significant effect on SAPK/JNK phosphorylation at lower concentrations \((10^{-9} \text{ M}-10^{-11} \text{ M})\). The control band showed constitutive activity.

Constitutive receptor is capable of producing its own biological response in the absence of a bound ligand. [75] Histamine receptor subtypes involved in SAPK/JNK phosphorylation were then determined. Specific H1, H2, H3, H4 histamine receptor agonists and antagonists were used. H1 receptor agonist betahistine \((10^{-5} \text{ M})\) suppressed SAPK/JNK phosphorylation. Decrease in SAPK/JNK phosphorylation by histamine was predominantly an H1 receptor effect. H1 antagonist pyrilamine \((10^{-6} \text{ M})\) partially reversed the effects of histamine on phosphorylation of SAPK/JNK. Stimulation of H1 receptor activates the \(G_q\) protein; H1 receptor activates phospholipase C and forms 1, 4, 5-triphosphate and 1, 2-diacylglycerol and increases cytosolic calcium levels. [80] Although H2 agonist amthamine \((10^{-5} \text{ M})\) did not show an effect on the phosphorylation of SAPK/JNK, its H2 antagonist ranitidine \((10^{-5} \text{ M})\) reversed the effects of histamine on SAPK/JNK phosphorylation. Ranitidine showed an effect even when amthamine had no effect on SAPK/JNK phosphorylation. This may be due to the fact that ranitidine may be acting as inverse agonist. [91] An inverse agonist binds to the same receptor binding-site as an agonist for that specific receptor and reverses the constitutive activity of that particular receptor. They exert the opposite pharmacological effect of a receptor agonist.
H2 receptor stimulation activates Gs protein and they couple to adenylate cyclase and increase cAMP production [80]. Zhang et al showed that cyclic AMP inhibited UV-induced apoptosis by suppressing JNK activation. [94] H3 agonist methimepip (10^{-6} M) augmented the phosphorylation of SAPK/JNK. H3 and H4 receptors both activate the G_{i/o} protein. H3 receptors inhibit cAMP formation and increase intracellular calcium levels and activate mitogen activated protein kinases. [80] Levi et al showed that the activation of H3 receptor caused MAPK phosphorylation in cardiac sympathetic nerves. They reported that the decrease of norepinephrine exocytosis is through MAPK activation. [79] H4 agonist 4-methyl histamine (10^{-6} M) also increased SAPK/JNK phosphorylation. H4 receptors also inhibit cAMP formation and induce calcium mobilization. [48]. Gutzmer et al reported that histamine via H4 receptor activated the transcription factor AP-1. AP-1, which is made of c-fos and c-jun, is a target for MAPK pathway. Using U0126, an AP-1 inhibitor, reversed the H4R mediated IL-12 suppression. This showed that histamine via H4 receptor activated JNK. [81] It is possible that in our system, H4 agonists are activating JNK by AP-1. H3/H4 antagonists thioperamide (10^{-6} M) and clobenpropit (10^{-5} M) also inhibited the effect of histamine on phosphorylation of SAPK/JNK. Thioperamide and clobenpropit also act as inverse agonists. [95, 96] Figure 9 shows the effect of histamine on SAPK/JNK phosphorylation in wild type mice.
Proinflammatory cytokines such as IL-1 beta and TNF-alpha play an important role in the inflammatory cascade in allergic asthma. [97] These cytokines are produced by the immune system and are critical mediators of shock after any trauma. TNF-alpha plays a critical role in asthma. Increased levels of TNF-alpha are seen in airway smooth muscle cells in asthmatic airways. They increase adhesion molecule expression which has an important role in recruiting inflammatory cells. [98, 99, 83] Barbin et al [84] explained that TNF-alpha triggers phosphorylation of SAPK/JNK. [84] Therefore, TNF-alpha knockout mice were used to determine whether histamine and its receptors work via TNF-alpha in SAPK/JNK phosphorylation. We found that histamine (10^{-4} M) inhibited SAPK/JNK phosphorylation in TNF-alpha knockout splenocytes. H1 agonist betahistine (10^{-5} M), H2 agonist amthamine (10^{-5} M), H3 agonist methimepip (10^{-6} M) down regulated the phosphorylation of SAPK/JNK in TNF-alpha knockout splenocytes. On the other hand, H4 agonist 4-methyl histamine (10^{-6} M), did not show any significant effect.
on the phosphorylation of SAPK/JNK in the absence of TNF-alpha. This suggested that increased phosphorylation of SAPK/JNK which involved H4 receptors was mediated by TNF-alpha. Hofstra et al [55] reported that histamine caused chemotaxis of mast cells. This chemotaxis was mediated via histamine H4 receptor. Also, H4 receptor activation caused calcium mobilization. Phospholipase C and G i alpha were involved in mast cell chemotaxis and calcium mobilization. They showed that this mechanism involving H4 receptor could be of use in allergic diseases like asthma. [55] Figure 10 depicts the effects of histamine and its receptors via other mechanisms on SAPK/JNK phosphorylation.

Coyne et al [100] showed that the JNK pathway is implicated in apoptosis of primary embryonic neurons when they were activated by various stimuli. This pathway was linked with other pathways which cause Alzheimer’s, Parkinson’s disease and stroke. Hence, JNK can play a vital role in CNS disease. [100] The JNK pathway is involved in
diabetes too. Kaneto et al [102] demonstrated that a new inhibitory JNK peptide was used, which increased insulin resistance in diabetic mice. [101] Kizilay et al [102] showed the involvement of the JNK pathway in proliferation and apoptosis in uterine endometrial cells. [102]

In summary, our data points to a role of H3 and H4 receptors in the secretion of TNF-alpha as it is involved in histamine mediated effects on SAPK/JNK phosphorylation. Wild type and knock out data are shown in the diagrammatic representation below. The study provides evidence that H4 antagonists may be clinically relevant in treating asthmatic inflammation which is difficult to treat with the exception of glucocorticoids.

![EFFECTS OF HISTAMINE ON SAPK/JNK PHOSPHORYLATION](image)

We compared and contrasted the effects of histamine on extracellular signal regulated kinases (ERK1/2) and stress activated protein kinases (SAPK). While histamine via H1
receptors inhibited SAPK/JNK phosphorylation, it increased its phosphorylation via H3 and H4 receptors. Histamine did not impact SAPK/JNK phosphorylation via H2 receptors. The results suggest that phosphorylation of SAPK/JNK by histamine via H1 and H2 receptors differed as opposed to ERK1/2 phosphorylation. However, in TNF-alpha knockout mice, the effects of histamine on SAPK/JNK and ERK2 phosphorylation were similar with the exception of the involvement of H1 and H4 receptors.
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