THE DETERMINATION OF THE AIRWAY RECEPTORS INVOLVED IN TRACHEA RELAXANT PROPERTIES OF AQUEOUS TIGER MILK MUSHROOM

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by

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LIST OF SYMBOLS, ABBREVIATIONS, AND ACRONYMS

%	Percentage
±	Plus, minus
µg	Microgram
µM	Micro Molar
AHR	Airway hyperresponsiveness
BALF	Bronchoalveolar lavage fluid
β2	Beta-2 adrenergic receptor
°C	Degree Celsius
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
Carbachol	Carbamylcholine chloride
cm	Centimetre
CO_2	Carbon dioxide
DRC	Dose-response curve
dH_2O	Distilled water
EC_{50}	Concentration of agonist that produces 50% maximal response
E_{max}	Concentration of agonist that has a maximal response
gm	Gram
GPT	Guinea-pig trachea
IC_{50}	Concentration of antagonists that produces 50% inhibition of the response of an agonist
IP	Intraperitoneal
KCl	Potassium chloride

kg	Kilogram
KH_2PO_4	Potassium hydrogen phosphate
L	Litre
M	Molar
M_1	Muscarinic acetylcholine receptor subtype 1
M_2	Muscarinic acetylcholine receptor subtype 2
M_3	Muscarinic acetylcholine receptor subtype 3
mAchR	Muscarinic acetylcholine receptor
mg	milligram
$MgSO_4$	Magnesium sulphate
ml	Millilitre
mM	Millimolar
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
nM	Nanomolar
O_2	Oxygen
pEC_{50}	The negative logarithm to base 10 of the EC_{50} of an agonist
pIC_{50}	The negative logarithm to base 10 of the IC_{50} of an antagonist
SEM	Standard Error Mean
WHO	World Health Organization
V	Volume

PENENTUAN RESEPTOR SALURAN PERNAFASAN DALAM KESAN PENGUCUPAN TRAKEA OLEH CENDAWAN SUSU RIMAU

ABSTRAK

Asma ialah “penyakit heterogen” yang telah dianggap sebagai kebimbangan terhadap kesihatan awam, yang biasanya dicirikan sebagai penyakit radang kronik saluran pernafasan, dan menghasilkan kesukaran bernafas secara berkala. Pendedahan kepada faktor pencetus seperti pemekaan alergen tersedut atau perengsa alam sekitar yang lain mengakibatkan bronkokostriksi pada lapisan tiub bronkial. Asma boleh dicirikan oleh halangan aliran udara boleh balik, keradangan saluran udara, rembesan mucus terlampau, dan tindak balas terlampau saluran udara. Cendawan Susu Rimau, yang juga dikenali sebagai *Lignosus rhinocerus* telah digunakan secara tradisional untuk ubat asma. Oleh itu, adalah penting untuk menentukan reseptor yang terlibat dalam mekanisme Cendawan Susu Rimau terhadap tindak balas terlampau saluran pernafasan. Objektif utama kajian ini adalah untuk menentukan reseptor saluran udara yang terlibat dalam Cendawan Susu Rimau terhadap sifat pengucupan dalam trakea tikus Belanda (GPT) terasing melalui kajian agonis-antagonis. Proses pengestrakan Soxhlet panas telah digunakan untuk menyediakan ekstrak Cendawan Susu Rimau. Kajian miograf berfungsi telah digunakan untuk membandingkan sifat pengucupan Cendawan Susu Rimau terhadap GPT terasing dengan antagonis tidak selektif muskarin, atropine pada tona pengucupan-karbakol (agonis tidak selektif muskarin). Cendawan Susu Rimau menunjukkan kesan pengucupan yang separa terhadap trakea tikus Belanda pada kepekatan yang lebih tinggi (1 g/ml) dalam 10 µM dengan pIC_{50} yang dihasilkan 1.42 ± 0.21. Atropin menghasilkan kesan pengucupan yang penuh pada 100 µM dengan pIC_{50} 3.91 ± 0.64. Kesimpulannya, kajian ini mendedahkan bahawa Cendawan Susu Rimau mempunyai sifat pengucupan terus pada trakea dan separa melalui reseptor muskarin.

THE DETERMINATION OF AIRWAY RECEPTORS INVOLVED IN TRACHEA RELAXANT PROPERTIES OF AQUEOUS TIGER MILK MUSHROOM

ABSTRACT

Asthma is "a heterogeneous disease" that has been considered a severe public health concern, usually characterized as a chronic inflammatory disease of the airways, and producing intermittent breathing difficulties. Exposure to trigger factors such as sensitizing inhaled allergens or other environmental irritants results in bronchoconstriction of the lining of the bronchial tubes. Asthma could be characterised by reversible airflow obstruction, airway inflammation, mucus hypersecretion, and AHR. Tiger milk mushroom, known as *Lignosus rhinoceros,* has traditionally been used for asthma medication. Therefore, it is essential to determine the receptor involved in the Tiger Milk Mushroom mechanism on airway hyperresponsiveness. The main objective of this study was to determine the airway receptors involved in trachea relaxant properties of aqueous Tiger Milk Mushroom in the isolated guinea-pig trachea (GPT) through an agonist-antagonist study. A hot Soxhlet extraction process was used to prepare Tiger Milk Mushroom extracts. In a functional myograph study, the isolated GPT was utilized to compare the relaxant properties of Tiger Milk Mushroom extract to the non-selective muscarinic antagonist, atropine, on carbachol-induced contractile (non-selective muscarinic agonist) tone. Tiger Milk Mushroom partially relaxed the carbachol-induced contraction of the isolated GPT at a higher concentration (1 g/ml) in 10 µM with established pIC_{50} of 1.42 ± 0.21. Atropine elicited complete relaxation at 100 µM with pIC_{50} of 3.91 ± 0.64. In conclusion, this study reveals that the Tiger Milk Mushroom partially possessed a direct tracheal relaxant through the muscarinic receptor.

Keywords: Asthma, Tiger Milk Mushroom, guinea-pig trachea, muscarinic receptor, myograph.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Asthma is one of the primary non-communicable diseases (NCD). It is defined as "a heterogeneous disease" usually characterized by chronic inflammatory disease of the airways (Hough *et al.*, 2020). Exposure to trigger factors such as sensitizing inhaled allergens or other environmental irritants results in bronchoconstriction of the lining of the bronchial tubes. Narrowing airflow in the lungs can lead to asthma as inflammation, and the tightening of the muscles causes contracted airways. According to the data reported by World Health Organization in 2019, asthma had affected an estimated 262 million people, and there were about 455 000 death cases in total. It has also become a significant non-communicable disease common among children and adults. Throughout the years, asthma-related death cases have been more expected among people from low-and lower-middle-income countries, where the possibility of getting a diagnosis and treatment becomes a tough challenge.

Asthma could be characterized by reversible airflow obstruction, airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (Muhamad *et al.,* 2019; Murdoch and Lloyd, 2010). Airway hyperresponsiveness (AHR) is considered a fundamental abnormality in asthma. In patients with an asthma attack, the airways will be swollen and inflamed, causing asthma exacerbation. This event will lead the smooth muscles around the airways to contract, leading to the overproduction of mucus and simultaneously triggering the lining of the bronchial tubes to narrow (Figure 1.1). Activation of some cells and mediators could also lead to a chronic inflammatory disorder in the airways, later causing asthma attacks. Therefore, asthma is closely associated with chronic inflammation and the influx of inflammatory proteins, which likely contribute to AHR (Chapman and Irvin, 2015). Acute asthma attacks can exhibit recurrent wheezing, cough, shortness of breath, and chest tightness which may lead to hospitalization or death (WHO, 2021).

Other than that, persistent structural changes in the airways associated with asthma broadly contribute to airway inflammation and airway wall remodeling (Keglowich and Borger, 2015). Airway inflammation is an essential factor in the chronic intermittent nature of asthma symptoms (Koziol-White and Panettieri, 2011), leading to increased airway hyperresponsiveness and eventually ending in severe asthma attacks (Malamed and Orr, 2015). As stated in the study by Holgate (2013), airway inflammation is characterized by increased production of inflammatory cells and mediators, including eosinophils, mast cells, and lymphocytes, with a predominance of Type 2 helper T lymphocytes that produce mediators such as interleukins. Ultimately, structural changes in airway remodeling can cause subepithelial fibrosis, increasing the smooth muscle mass, gland enlargement, neovascularisation, and epithelial changes in both large and small airways (Bergeron *et al.,* 2010). In the context of such airway disorders, airway smooth muscle (ASM) mainly regulates airway contraction and structural remodeling of the bronchial wall, leading to the development and progression of asthma (Pelaia *et al.,* 2008).

Since asthma becomes a complex disease process with no cure, finding an alternative treatment to improve asthma attacks in asthma patients should focus on understanding the underlying physiology and pathophysiologic mechanisms. Numerous medications in clinical settings can treat or relieve (reliever medication) and control (controller medication) symptoms and reduce (preventer medication) asthma exacerbations. However, these preventive medicines mainly use steroids and other anti-inflammatory drugs, which inappropriately cause adverse effects (Mukherjee *et al.,* 2017). Asthma control medications broadly help in reducing airway inflammation and are suitable for preventing asthma symptoms. Long-term control medications could help relieve airway inflammation and hyperresponsiveness, mainly preventing the underlying symptoms from worsening. Leukotriene receptor antagonists can efficiently control airway inflammation and hyperresponsiveness. Montelukast and zafirlukast are antagonists for the pro-inflammatory leukotriene receptor in the inflammatory response and use as preventive medicine for asthma. Other than that, quick-relief medications quickly ease the underlying symptoms that may arise acutely during an asthma attack. Among these, short-acting beta-agonists (SABAs) can rapidly reduce airway bronchoconstriction, which relaxes airway smooth muscles (Papi *et al.,* 2020).

Adrenergic agonists such as salbutamol, terbutaline, and formoterol act on β2-adrenoceptor to relax the contracted airways. Anticholinergic agents, including atropine, oxybutynin, and darifenacin, act as antagonists to muscarinic receptors. Therefore, combining muscarinic receptor antagonists and β-adrenergic agonists in asthma medications can improve airway disorders among patients with chronic asthma.

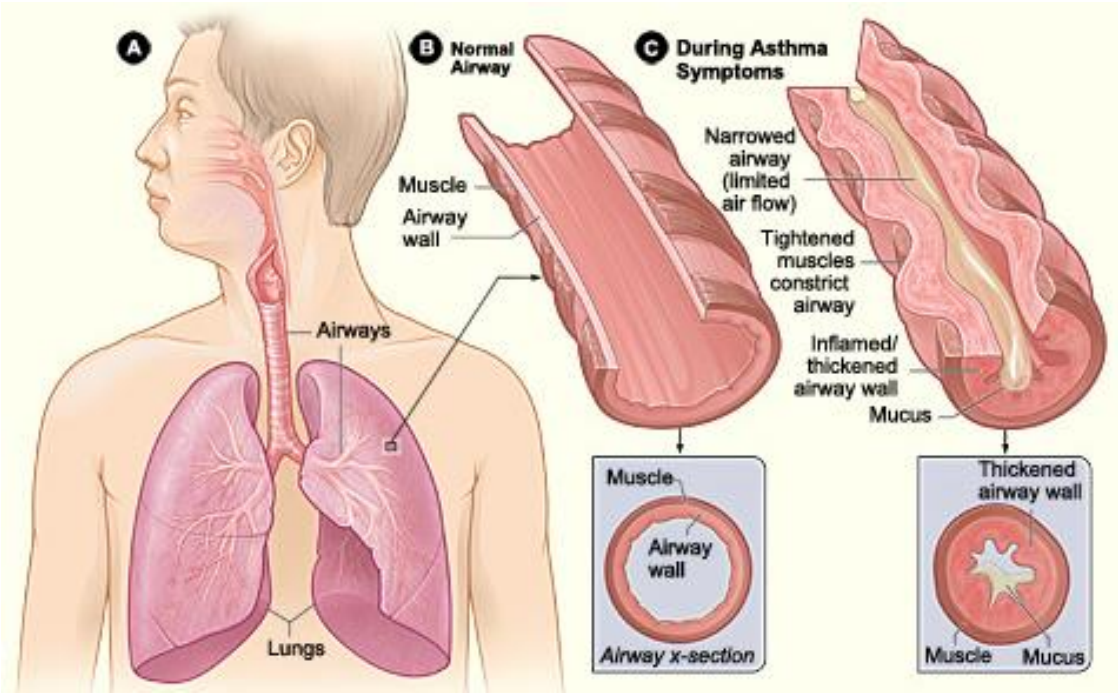


Figure 1.1 The pathophysiology of asthmatic airways during asthma exacerbations (Sinyor and Concepcion Perez, 2022)

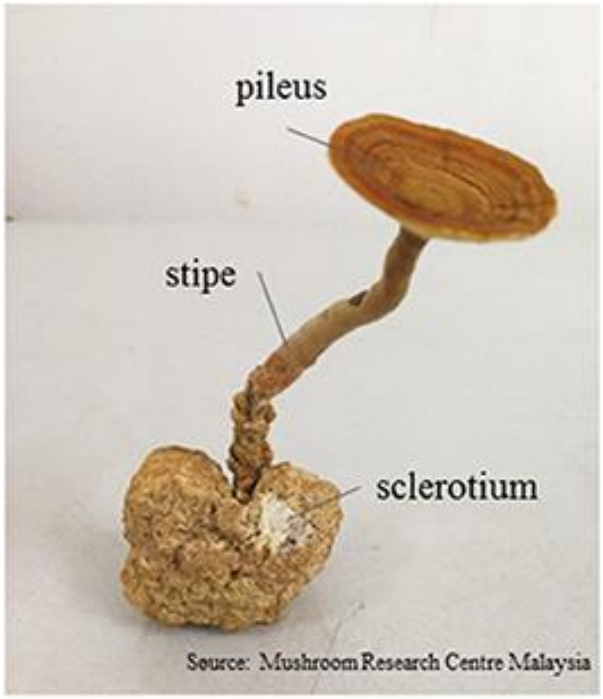
1.2 Tiger Milk Mushroom

Tiger milk mushroom, scientifically known as *Lignosus rhinoceros*, is a polypore fungus belonging to the Polyporaceae family and the order of Polyporales, Basidiomycota. The mushroom consists of three parts, pileus as its cap, the stem called stipe, and the tuber known as a sclerotium, which is the part that can encompass medicinal properties (Figure 1.2). Tiger milk mushroom is one of the most valued fungi used widely for traditional medication in Southeast Asia and South China. Nallathamby *et al.* (2018) stated that the species is locally known as "cendawan susu rimau" in Malaysia after a spot where a tigress drops milk while feeding.

The sclerotium part of the mushroom provides antioxidant, antimicrobial, anti-inflammatory, and anti-asthmatic effects and even can be good in enhancing immunomodulatory activities (Johnathan *et al.,* 2016; Lau *et al.,* 2014; Lee *et al.,* 2014 & Mohanarji *et al.,* 2012). A previous study by Lee *et al.* (2012) demonstrated that the mushroom extract of sclerotia could alleviate illnesses like breast cancer, fever, cough, asthma, and food poisoning. According to Lee *et al.* (2009), the Temuan tribe in Malaysia was able to treat cough and asthma by boiling the sclerotium part of the mushroom. The sclerotium part of this mushroom is also highly hunted by the native communities to avert hunger, soothe cough and asthma, and offer stamina (Nallathamby *et al.,* 2018). Indigenous communities in Peninsular Malaysia have also traditionally exploited the mushroom as their natural medication for treating various ailments, including respiratory illnesses (Tan *et al.,* 2021). It was stated in the study by Yap *et al.* (2013) that natural compounds found in Tiger Milk Mushroom, such as polysaccharides and polysaccharide-protein complexes, can potentially modulate the human immune system.

Multiple *in vitro* and *in vivo* studies showed that the Tiger Milk Mushroom extracts effectively relieve asthma. Oral and intranasal administration of Tiger milk mushroom extracts attenuates asthmatic parameters. It could significantly lower the Th2 cytokines level in the bronchoalveolar lavage fluid (BALF), the level of IgE in the serum, and the leukocyte number from infiltrating the lung tissues (Johnathan *et al.,* 2016; Muhamad *et al.,* 2019). To add, a recent study by Tan *et al.* (2021) validated that the

Tiger Milk Mushroom extracts effectively reduced the infiltration of eosinophil into the lungs of ovalbumin (OVA)-sensitized asthmatic rats. Thus, it proved that Tiger Milk Mushroom could be an alternative treatment to treat acute asthma and reduce the inflammation of airways. However, very scarce scientific evidence has testified to the effect of exploiting the bioactive fractions or compounds of Tiger Milk Mushroom to mediate airway relaxation during asthma exacerbations.



Kingdom : **Fungi**

Phylum : **Basidiomycota**

Class : **Agaricomycetes**

Order : **Polyporales**

Family : **Polyporaceae**

Genus : ***Lignosus***

Species : ***Lignosus rhinocerotis* (Cooke) Ryvarden**

Figure 1.2 The morphology of Tiger Milk Mushroom (*Lignosus rhinocerus*) and the classification of its taxonomy (Nallathamby *et al.*, 2018).

1.3 Justification of the study

Asthma causes occasional breathing difficulties, considered a severe public health concern. There is currently no cure, but many simple treatments can help manage the underlying symptoms that could impact a patient's life. However, there was a claim that most asthma medications are not cost-effective and could cause many side effects to the patients. Therefore, as dietary herbs and medicinal plants grew interested in clinical fields, there were extensive discoveries on alternatives for asthma treatments.

Several animal models of asthma have been created so that relevant pathological mechanisms can be studied. For example, mice have been used for a long time to figure out how respiratory diseases cause inflammation. However, the mediators that control smooth muscle tone and determine bronchial responsiveness in mice are very different from those in humans (Canning, 2003). Also, the murine pulmonary structure is characterised by large and sparse airways in smooth muscle tissue (Zosky and Sly, 2007) and less responsiveness to several bronchoconstrictor stimuli involved in the pathophysiology of asthma.

Johnathan *et al.* (2016) have shown that the Tiger Milk Mushroom extracts worked on inhibiting AHR, performed in asthmatic Balb/c mice induced with house dust mite (HDM). In addition, Tiger Milk Mushroom effectively reduced airway inflammation of ovalbumin-induced allergic asthma in Sprague-Dawley rats. However, although numerous asthmatic animal models have been used, most models focus on short-term exposure to allergens that cause acute inflammation. Also, many models fail to display the features of human airway hyperresponsiveness. In this study, the Guinea pig trachea was used to mimic the characteristics of contractile hyperreactivity and relaxant hyperresponsiveness of airway smooth muscle, similar to those seen in human asthma.

Tiger Milk Mushroom could be one of the therapeutic potentials as a new model of asthmatic treatment against allergic asthma since it can alleviate allergy airway inflammation and hyperresponsiveness. Furthermore, this study may be a valuable tool for studying the mechanisms of AHR and finding new drugs to treat asthma. However, a limited scientific study has proven the efficacy of Tiger Milk Mushroom extracts in relieving airway inflammation and hyperresponsiveness in another asthmatic model. Therefore, this study is designed to determine which receptors may involve in the Tiger Milk Mushroom mechanism for asthma relief in the isolated trachea of the Guinea pig.

1.4 Objectives of the study

This study aims to determine the airway receptors involved in trachea relaxant properties of aqueous Tiger Milk Mushroom through an agonist-antagonist study.

The specific objectives of this study include the following:

i. To determine the effect of muscarinic receptor agonist (carbachol) on the isolated Guinea-pig trachea (GPT).
ii. To determine the muscarinic receptor antagonist (atropine) effect against carbachol on the isolated GPT.
iii. To determine the relaxation effect of Tiger Milk Mushroom extracts against carbachol on the isolated GPT.

1.5 Scope of the study

This study determines the receptors responsible for Tiger Milk Mushroom's relaxation effects in GPT. In addition, this study evaluates the dose-response curve of Tiger Milk Mushroom and its relaxant properties in airway smooth muscles. Thus, this study hypothesized that Tiger Milk Mushroom would be able to evaluate its roles in the airway smooth muscle relaxation mechanism through muscarinic receptors.

CHAPTER 2

LITERATURE REVIEW

2.1 Pathophysiology of asthma

By definition, asthma is a chronic relapsing airway disorder that can cause the airways in the lungs to become constricted. Asthma involves a complex interaction of airway obstruction, bronchial hyperresponsiveness, and an underlying inflammation. The degree of airway inflammation is more likely associated with asthma severity and hyperresponsiveness. Inflammation of the airways is characterized by acute asthmatic inflammation featuring primary recruitment of inflammatory cells into the airway, subacute asthmatic inflammation that involves activation of recruited cells in recurrent inflammation, and chronic inflammation, which is characterized by cellular damage (Arora and Ansari, 2019).

Persistent airway limitation in asthma can cause various changes in the airway. These include bronchoconstriction, hyperresponsiveness, mucus hypersecretion, airway edema, and ultimately airway obstruction and remodeling, which is typical of asthma. In chronic asthma exacerbations, airway smooth muscle contraction can narrow the airways in response to various internal and external stimuli, including allergens, which could lead to an airway inflammatory response (Figure 2.1). Allergen-induced chronic airway smooth muscle constriction results from an IgE-dependent activation of airway mediators from mast cells, including histamine, leukotrienes, and prostaglandins, which could directly contract the airway smooth muscle (Busse and Lemanske, 2001). Airway mast cells can be a vital source of small proteins, including cytokines and chemokines associated with the allergic response, such as interleukin. In addition, these small proteins, combined with inflammatory mediators, can stimulate the recruitment and activation of secondary effector cells, neutrophils, eosinophils, and T lymphocytes to induce late-phase airway narrowing and AHR (Holgate, 2013). Therefore, certain inhaled stimuli like environmental allergens could increase airway inflammation and enhance airway hyperresponsiveness.

In addition, other stimuli, including cold air or irritants, can also lead to acute airflow obstruction. Edema, inflammation, hypersecretion of mucus, and structural changes, including hypertrophy and hyperplasia of the airway smooth muscle, could further limit airflow as the disease becomes more recurrent and inflammation become more progressive. An abnormal accumulation of inflammatory cells in the bronchioles can lead to airway inflammation. Repetitive airway inflammation could result in airway damage, including hypertrophy of smooth muscle, epithelial hyperplasia, and airway connective tissue becoming deposited (Salyer, 2007). Gordon *et al.* (2014) stated that the involvement of genes had been considered in the context of environmental influences on asthma development and pathogenesis. As multiple genes transmit asthma, it can cause the variation of locus heterogeneity and polygenic inheritance, leading to complex asthma expression (Sinyor and Concepcion Perez, 2022).

Following exposure to inhaled allergens, parasympathetic nerves are the vital factor that controls the lung, which increases the contraction of airway smooth muscles. In asthma, parasympathetic nerves supply the lung, maintain airway tone, and play a prominent role in airway narrowing and mucus secretion (Scott and Fryer, 2012). Parasympathetic nerves control the symptoms and inflammation of airways by the involvement of the peripheral muscarinic receptors. Parasympathetic neural activity stimulates effector tissues like airway smooth muscle and secretory glands to mediate acute asthmatic symptoms, including airway obstruction and mucus hypersecretion (Scott and Fryer, 2012).

Acetylcholine (Ach) is the predominant parasympathetic neurotransmitter in the airways. Therefore, amplifying acetylcholine binding in parasympathetic neural activity indicates the pathophysiology of asthma (Kistemaker and Gosens, 2015). According to Gosens and Gross (2018), releasing acetylcholine by airway neurons and binding it to the muscarinic receptors leads to smooth muscle contraction. In addition, it increases mucus secretion, inflammation, and airway remodeling (Figure 2.2). Therefore, acetylcholine promotes inflammation and remodeling by direct effects on airway cells, and mechanical stress applied to the airway progressive to smooth muscle constriction.

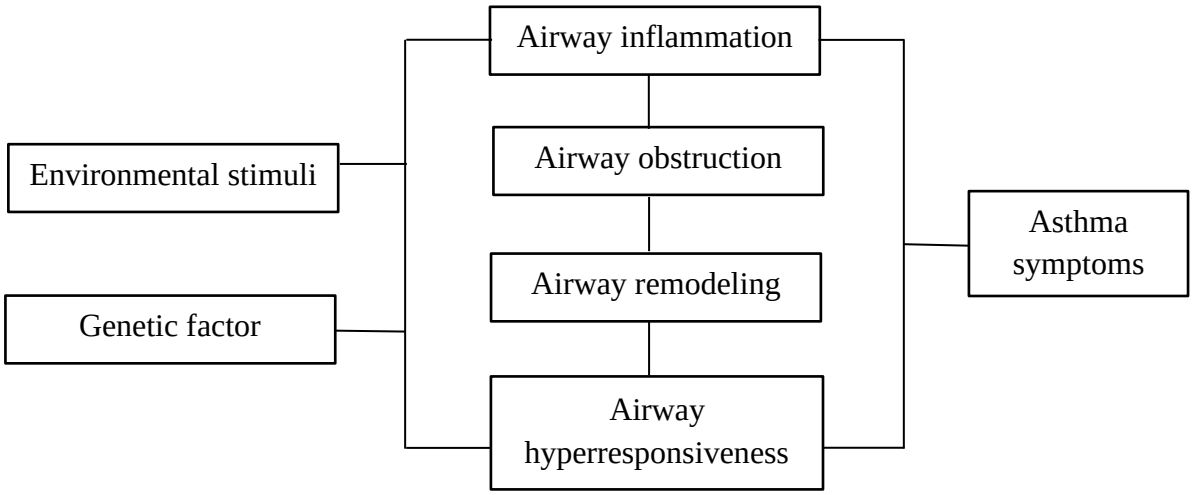


Figure 2.1 Pathophysiology of asthma.

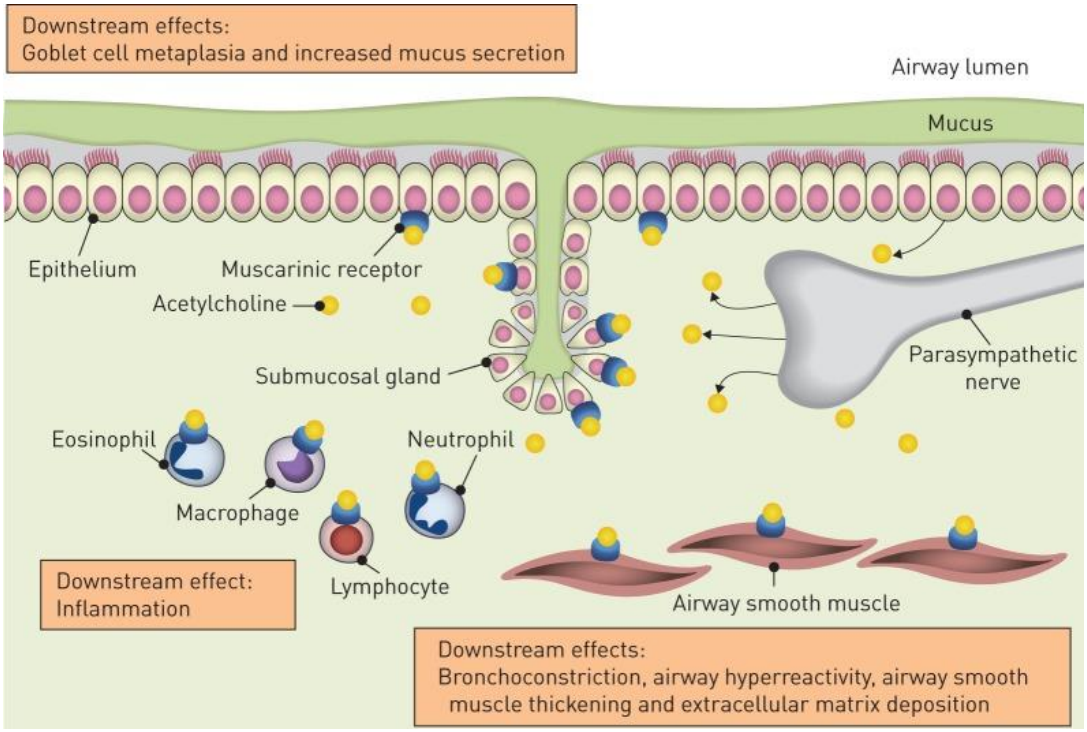


Figure 2.2 The role of acetylcholine in asthma pathophysiology (Gosens and Gross, 2018).

2.2 Asthma medications

Asthma is a broad-spectrum, chronic inflammatory disease with millions of everyday sufferers is asthma. It is brought on by an improper inflammatory response, such as those brought on by inhaled environmental allergens, ultimately contributing to bronchial hyperreactivity constriction, smooth muscle remodeling, and increased mucus secretion into the airways. Therefore, many medications treat and manage chronic asthma to relieve symptoms and decrease asthma exacerbations. The medicines include leukotriene receptor antagonists, long-acting beta-agonists, anticholinergics, low to high-dose inhaled corticosteroids, beta-2 agonists, and monoclonal antibodies immune-modulating therapies (Sharma, Hashmi and Chakraborty, 2022). In addition, corticosteroids that are taken orally are convenient in the acute improvement of asthma exacerbations.

Treatments for obstructive airway disease include muscarinic receptor antagonists and β-adrenoceptor agonists. Muscarinic receptors and β-adrenoceptors are physiological antagonists that work against each other to keep smooth muscles in the airways from getting too tight. Muscarinic agonism inhibits β-adrenoceptor-mediated relaxation more than by other contractile stimuli. The expression of both the target and the opposite receptor may be changed by the long-term use of one drug class. Prejunctional β2-adrenoceptors can make it easier for neurons to release acetylcholine (Dale *et al.*, 2014).

β2-adrenoceptor agonists are a class of medications utilized as a mainstay in the frontline management and treatment of bronchial asthma. The available β2-adrenoceptor agonists like bronchodilators are believed to act on beta-2 airway receptors, which are used to relax airway smooth muscles and enhance airflow most of the time, lowering lung hyperinflation (Albertson *et al.*, 2015). β2-adrenoceptor agonists can mimic the actions of catecholamines like adrenaline, norepinephrine, and dopamine in triggering various autonomic responses in the body. The circulating catecholamines through the autonomic system can trigger parasympathetic and sympathetic physiological reactions. β2 agonists have a higher affinity for ß2-adrenergic receptors and would function as ligands for adrenergic receptors by mimicking catecholamines. β2-adrenoceptor agonists impact the smooth muscle of the

airway, uterus, gut, and systemic vasculature (Hsu and Bajaj, 2022). The classification of β2 agonists involves short-acting beta-agonists (SABAs) and long-acting beta-agonists (LABAs). As the names suggest, SABAs have the shortest half-life and provide instant relief from asthmatic symptoms, whereas LABAs, with their longer half-lives, provide treatment that lasts for more extended periods.

For acute treatment of asthma symptoms and exacerbations, SABAs are the preferred medications. In the treatment of asthma, SABAs are also frequently used with LABAs, inhaled corticosteroids, or long-acting muscarinic agonists. These drugs are often administered via metered dose inhalation or dry-powder inhalation. Inhalation offers a higher therapeutic efficacy and fewer systemic adverse effects than alternative oral administration (Barisione *et al.,* 2010). Salbutamol is the hallmark of SABAs that has an onset of action in less than five minutes and a duration of therapeutic efficacy between 3 and 6 hours. Other than that, salbutamol (albuterol) is most commonly used as an airway treatment.

LABAs are frequently combined with inhaled corticosteroids to treat patients with acute asthma. Recent research by Rodrigo *et al.* (2017) has shown that dual therapy is more effective than monotherapy LABA. Compared to SABAs, LABAs have an onset duration longer than five minutes, with up to 15 minutes for salmeterol, and their duration of effect is at least 12 hours. Like SABAs, inhalation is the suggested administration route to consuming class medications of LABAs. LABAs are the preferred add-on medication to inhaled corticosteroids and second-line treatment in relieving asthma exacerbations (Albertson *et al.,* 2015).

Numerous developing medications through technical advancement are focused on targeting various inflammatory pathways. Novel and emerging herbal products are thought to work on particular mechanistic pathways in asthma. Following asthma treatment, the standards for determining the most effective therapy for persistent asthma management should be patient-centred and adhere to the authority's suggested policy. Environmental factors should also be measured to reduce exposure to stimuli, and relevant prevention approaches should be implemented. Therefore, the development of these medication classes should be focused primarily on the therapeutic consequences of their potential to manage asthmatic symptoms.

2.3 Muscarinic receptor regulation in airway smooth muscle

Muscarinic receptors are G-coupled protein receptors essential to the parasympathetic nervous system. Muscarinic receptors derive their name from their elevated sensitivity to muscarine, a compound discovered in certain mushroom species (Jin, 2016). In addition, the acetylcholine molecules can trigger muscarinic receptors to facilitate a parasympathetic response in organs and tissues where the receptor is expressed (Kudlak and Tadi, 2022). Acetylcholine is closely linked to muscarinic receptors and parasympathetic nervous system activation. Sweat glands, however, are part of the sympathetic response and are not affected by acetylcholine. Excessive stimulation of these receptors can increase the parasympathetic response, bringing other adverse effects. Malfunctioning muscarinic receptors are associated with various ailments. For example, overstimulation of the parasympathetic nervous system leads to COPD and asthma caused by tightening airways, leading to difficulty breathing and shortness of breath.

As asthma dyspnea symptoms are caused by bronchoconstriction and smooth muscle constriction, this constriction can be attributed to increased parasympathetic activity, which includes overstimulation of muscarinic receptors via increased acetylcholine release (Buels and Fryer, 2012). In addition, increased acetylcholine release in asthmatic airway inflammation is related to increased ASM contractility, mucus hypersecretion, and improved parasympathetic tone (Gosens *et al.,* 2006).

Muscarinic receptors play a role in peristalsis, micturition, bronchoconstriction, and several other parasympathetic responses (Albertson *et al.,* 2020; Kozlova *et al.,* 2019 & Patel *et al.,* 2020). Muscarinic receptors are ligand-gated G-protein coupled receptors that work as either stimulative regulative G-proteins (Gs) or inhibitory regulative G-proteins (Gs) (Gi). For example, Ca^{2+} is activated by Ach stimulation of Gq. There are five subtypes of receptors that, when dysfunctional or overstimulated, can be targeted by several medications for symptom relief. Muscarinic receptors control smooth muscle tone, mucus secretion, vasodilation, and inflammation in healthy lungs. M_1, M_2, M_3, M_4, and M_5 are subtypes of muscarinic receptors recognized by the International Union of Pharmacology (Caulfield and Birdsall, 1998). All five muscarinic receptor subtypes are expressed in the lungs. However, only M_1, M_2, and

M_3 receptors contribute to airway physiology and diseases such as asthma and COPD (Gosens and Gross, 2018). It also stated that M_2 and M_3 muscarinic receptor subtypes are abundantly expressed by airway smooth muscle, roughly around a 4:1 ratio (Gosens *et al.*, 2006a). In Guinea pigs, M_1 muscarinic receptors can depolarize the resting membrane potential around ganglion cells, which would be predicted to promote neurotransmission at the synapse and lead to bronchoconstriction (Myers and Undem, 1996). M_3 receptors primarily regulate airway smooth muscle contraction, whereas M_2 receptors work by preventing beta-adrenergic-stimulated relaxation (Hirshman, Lande and Croxton, 1999). As a result, the presence of eosinophils in the airway is enhanced in asthma. Eosinophils secrete an essential basic protein that binds to M_2 receptors. The binding of M_2 receptors causes acetylcholine cannot attach to the receptors, making airway relaxing difficult; this permits unbound acetylcholine to bind to M_3 receptors, triggering airway constriction and creating asthma symptoms (Buels and Fryer, 2012).

In asthma, on the other hand, cholinergic mechanisms contribute to increased bronchoconstriction and mucus hypersecretion, which limit the airflow. The cholinergic system controls numerous pathophysiological pathways. Through muscarinic receptors, the vagal parasympathetic nervous system exerts the predominant autonomic control over airway smooth muscle tone in the lung. The production of acetylcholine at neuromuscular junctions can bind to M_3 muscarinic receptors in smooth muscle and stimulates airway contraction via various distinct intracellular signaling mechanisms (Fryer and Jacoby, 1998). The neuronal M_2 muscarinic receptor, which limits acetylcholine release, provides essential negative feedback (Coulson and Fryer, 2003). The loss of function of vagal M_2 receptors due to eosinophilic inflammation is a suggested reason for allergic AHR to methacholine (MCh). Cholinergic agonists such as methacholine often cause airway smooth muscle constriction in asthmatic patients. It is primarily regulated by muscle muscarinic M_3 receptors and adversely by vagal muscarinic M_2 receptors (Castro *et al.*, 2013). Therefore, blocking M_2 receptors causes an increase in the release of acetylcholine from vagal nerve terminals, which ultimately promotes bronchoconstriction (Belmonte, 2005).

Though the Gq-coupled M_3 receptor has a lower expression level, it is the primary muscarinic subtype in charge of the bronchial and tracheal smooth muscle contraction. It was proven by the fully functioning affinities of varied subtype-selective antagonists in airway tissues from various species, including humans (Roffel *et al.*, 1990, 1988; Ten Berge *et al.,* 1993 & van Nieuwstadt *et al.,* 1997). Nevertheless, as stated in pharmacological research by Struckmann *et al.* (2003), Gi-coupled M_2 receptors have a limited role in regulating airway smooth muscle contraction in peripheral airways. In asthma, muscarinic receptor regulation of airway smooth muscle tone is augmented by specific mechanisms, including an increased expression and function of signaling molecules vital for mediating muscarinic receptors and airway smooth muscle contraction. And second is the exaggerated release of neuronal acetylcholine due to neuronal mechanisms associated with inflammation (Gosens *et al.,* 2006).

Muscarinic receptors can help promote airway smooth muscle contraction via various intracellular signaling mechanisms (Gosens *et al.,* 2004). In addition, several studies have suggested that the increased Ca^{2+}signaling contributes to the development of obstructive airway diseases associated with airway hyperreactivity (Amrani and Panettieri, 2002). Muscarinic receptors promote smooth muscle contraction in the airways via Ca^{2+}-dependent and Ca^{2}-independent pathways (Figure 2.3). Ca^{2+}-independent mechanisms can be specified by enhanced contraction at a constant Ca^{2+} concentration, a phenomenon called Ca^{2+} sensitization (Gosens *et al.,* 2006). Thus, M_2 and M_3 muscarinic receptors can activate a critical metabolic pathway of Ca^{2+} sensitivity in airway smooth muscle (Lutz *et al.,* 2005). Furthermore, muscarinic receptor-linked signaling in regulating Ca^{2+} sensitivity of airway smooth muscle cells seems likely to be augmented in experimental models of inflammatory airway disorder.

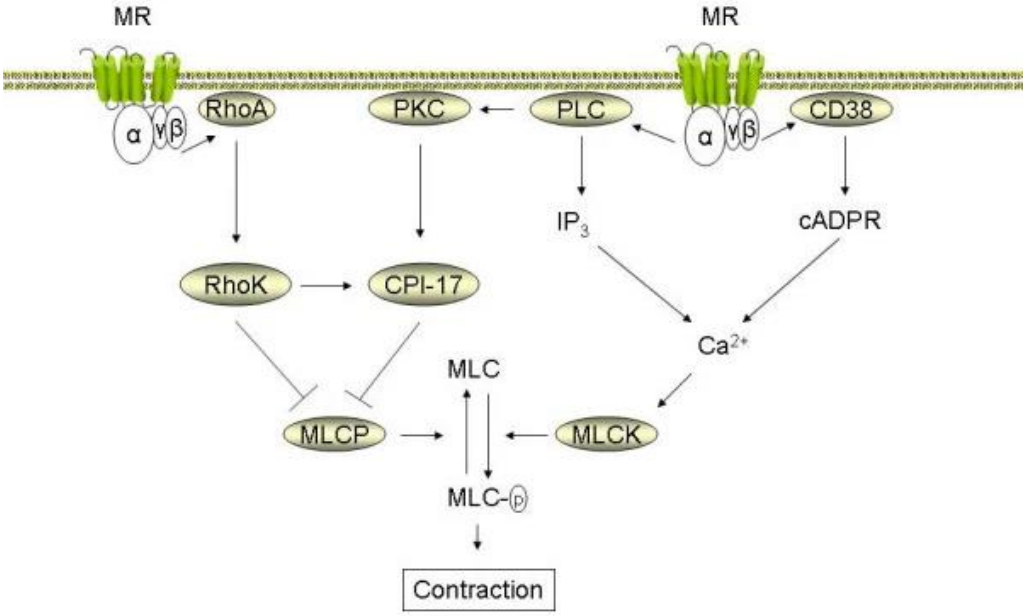


Figure 2.3 Central pathway in muscarinic receptor-mediated airway smooth muscle contraction (Gosens *et al.*, 2006).

2.4 Muscarinic receptors function in asthma

2.4.1 Airway inflammation

Airway inflammation has emerged as a significant factor in asthma pathogenesis. Inflammation of the airways is prevalent even in the absence of acute symptoms. Inflammation of the airways is the primary cause of the persistent intermittent pattern of asthma symptoms, which ultimately leads to severe asthma attacks (Gillissen and Paparoupa, 2015). Although the inflammation affects all airways, from the upper respiratory tract to the small airways, its physiological effects are thought to be most apparent in medium-sized bronchi. Therefore, the inflammation observed in asthma is primarily localized in the larger conducting airways. Small airways can be impacted in more severe diseases, but the lung parenchyma is unaffected.

In addition, chronic respiratory tract inflammation and augmented inflammation are closely related to asthma exacerbations. Chronic inflammation of the airways is like a feature of asthma, but inflammation patterns are markedly different as inflammation involves the proximal airways. In the inflammatory process, eosinophils, mast cells, and other subtypes of T cells play a significant role in causing asthma. A study by Barnes (2008) has asserted that the regulation of airway inflammation is by the T

helper (Th) type 2 cytokines, IL-4, IL-5, and IL-13. The number of $CD4^+$ T cells in the airways increases in asthmatic patients, and these are essentially T helper 2 (Th2) cells, whereas Th1 cells predominate in normal airways (Meyer, DeKruyff and Umetsu, 2008). Th2 cells play an essential role in allergic inflammation by secreting the cytokines IL-4 and IL-13, which promote IgE synthesis by B cells, IL-5, which is solely responsible for eosinophil development in the bone marrow, and IL-9 attracts and drives mast cell differentiation (Kay, 2006). Th2 cells play a dominant role in allergic inflammation; hence, their modulation is a subject of intensive study.

In asthma, other than T helper 2 (Th2) ($CD4^+$) cells predominate, the activation of mucosal mast cells infiltration of eosinophils has also been regulated. So, the inflammation that occurs in asthma can be defined as eosinophilic. Eosinophils are the key feature that plays a vital role in the pathogenesis of asthma by releasing inflammatory mediators (Nakagome and Nagata, 2011). Therefore, several mechanisms are involved in the eosinophilic airway inflammation of asthma. In the pathogenesis of acute asthma, not only eosinophils are involved, but also mast cells or neutrophils. Eosinophils have long been thought to be central effector cells in allergic airway inflammation. Due to their ability to release cationic proteins, which are harmful to the epithelium, other mediators, such as the cysteinyl leukotrienes, have made eosinophils a key marker of asthmatic inflammation and, more recently, a therapy target for IL-12 and anti-IL-5. (Bryan *et al.*, 2000; Leckie *et al.*, 2000).

Gosens *et al.* (2006) explained that muscarinic receptor-linked signaling pathways modulate Ca^{2+} sensitivity of airway smooth muscle cells in experimental models of the inflammatory airway. Muscarinic receptors on airway smooth muscle cells may play a significant role in regulating airway inflammation. A study shows that the muscarinic receptor agonist carbachol promotes inflammatory gene transcription in bovine tracheal smooth muscle strips. This finding supports the hypothesis that muscarinic receptors on airway smooth muscle cells significantly regulate airway inflammation (Kanefsky *et al.,* 2006). Recent evidence also reveals that bronchoconstriction can regulate airway inflammation and remodeling, implying that the muscarinic M_3 receptor plays a role (Kistemaker *et al.,* 2014). Furthermore, acetylcholine has been proven to produce airway smooth muscle contraction via the muscarinic M_3 receptor and to generate airway inflammation via muscarinic receptors. Airway remodeling and

inflammation are related to the expression of muscarinic Ach receptor (mAchR) subtypes (M_1, M_2, and M_3).

Acetylcholine is not traditionally thought to be an inflammatory mediator. Nonetheless, current research indicates that, in addition to choline acetyltransferase (ChAT), acetylcholine is expressed not only in neurons but also in inflammatory cells, including lymphocytes, macrophages, mast cells, eosinophils, neutrophils, in which infiltrating bronchial tissue during chronic inflammatory disorders such as asthma (Koarai and Ichinose, 2018). Furthermore, stimulation of muscarinic M_3 receptors effectively can increase cytosolic Ca^{2+} levels in human T- and B-cells and may influence T-cell cytotoxicity; yet, activation of lymphocytes enhances the expression and muscarinic receptors (Gosens *et al.,* 2006). Acetylcholine may play a role in airway inflammation via autocrine/paracrine mechanisms.

The regulation of airway inflammation by muscarinic receptors is further supported by discoveries that non-neuronal acetylcholine is extensively produced by resident cells, such as epithelial cells, in response to inflammatory stimulation in human bronchi (Cazzola *et al.,* 2016; Calzetta *et al.,* 2018; Rogliani *et al.,* 2021). Intriguingly, activation of muscarinic receptors expressed on small airway epithelium causes the release of chemotactic molecules and, as a result, the migration of eosinophils, monocytes, and neutrophils into bronchial tissue (Gosens *et al.,* 2006). Overall, these pathways related to non-neuronal acetylcholine activation of muscarinic receptors are associated with inflammatory cell proliferation and enhanced cytokine production, leading to harmful inflammatory consequences in asthma. Notably, muscarinic receptors expressed on ASM cells may support or trigger airway inflammation via the transcription of pro-inflammatory genes produced by increased sinusoidal length oscillation due to altered breathing cycles, a recurrent phenomenon in chronic obstructive respiratory illnesses (Kanefsky *et al.,* 2006). In addition, the inflammatory response may contribute to airway remodeling, causing morphological changes such as goblet cell metaplasia, airway smooth muscle thickening, and subepithelial fibrosis. Furthermore, pulmonary vascular remodeling and enhanced angiogenesis are mainly related to increased vascular endothelial growth factor (VEGF) release (Hoshino, Nakamura and Hamid, 2001; Jeffery, 2001).

The effectiveness of muscarinic receptor antagonists in reducing airway inflammation has been demonstrated in studies ranging from the laboratory to the clinic. In addition, an accumulating suggestion has indicated that long-acting muscarinic antagonists (LAMAs) may affect airway contractility and hyperresponsiveness not only by blocking mAchRs expressed on the airway smooth muscle but also by blocking mAchRs expressed on inflammatory cells, submucosal glands, and epithelial cells via anti-inflammatory processes (Calzetta *et al.,* 2021). Furthermore, non-neuronal Ach produced by airway epithelium regulates ASM contractility in small airways and possibly inflammation, with LAMAs mitigating these harmful effects. However, there have been no systematic reviews of the anti-inflammatory effect of muscarinic receptor antagonists in respiratory medicine.

2.4.2 Airway hyperresponsiveness

Hyperresponsiveness of the airways has long been considered a cardinal feature of asthma, and its measurement has provided profound insights into the underlying pathophysiology of asthma (Chapman and Irvin, 2015). AHR in response to direct and indirect stimuli is a universal and defining feature of asthma (Cockcroft and Davis, 2006). Airway hyperresponsiveness (AHR) is well-defined as the predisposition of the airways to narrow excessively in response to stimuli that would produce little or no effect in healthy individuals. AHR consists of an amplified airway sensitivity to an inhaled constrictor agonist, a steeper slope of the dose-response curve, and a better maximal response to the agonist (O'Byrne and Inman, 2003). Moreover, AHR can be measured to diagnose asthma, predominantly in patients with symptoms consistent with asthma and without evidence of airflow obstruction.

Airway hyperresponsiveness becomes a tool in the diagnosis, classification of severity, and management of asthma (Woolcock *et al.*, 1985; Sont *et al.,* 1999; Fowler *et al.,* 2000). A study by Brutsche *et al.* (2006) indicated that AHR is associated with an increased decline in lung function; even those with asymptomatic AHR would increase the risk of asthma development and an increased likelihood of persistent wheezing from childhood to adulthood. AHR has a variable component caused by acute inflammatory events and a chronic component caused by chronic inflammation. These components can result in structural and phenotypic changes to the airway smooth

muscle. AHR is more likely due to an increased release of mediators from inflammatory cells, such as histamine and leukotrienes. The release of these mediators would directly cause smooth muscle constriction and enhance bronchoconstrictor response to other agonists (Meurs, Gosens and Zaagsma, 2008).

Despite much research, there is still minor consent on the mechanisms underlying AHR in asthma. This mechanism is most likely due to the various pathophysiological abnormalities associated with asthma and the possible reality that different mechanisms of these give rise to AHR in other patients.

2.4.3 Mucus hypersecretion

Cholinergic activity is responsible for controlling mucus formation in the central airways, which is a crucial component in the pathophysiology of asthma (Rogers, 2001). The mucus in the airways acts as a barrier that protects inhaled particles from causing damage to the epithelium lining the airways. Airway mucus comprises electrolytes and water and has many mucins in its structure (Rogers, 2004). The high viscosity of the mucus is caused by the glycoproteins known as mucins. Mucus is a by-product of the immune system. Goblet cells, which are found embedded in the epithelium, and submucosal glands, which are attached to the airway lumen, produce mucus in the central airways.

In the central airways, acetylcholine is the primary neurotransmitter involved in mucus secretion (Rogers, 2001). This vagally controlled mucus production is most likely sourced from airway submucosal glands. Submucosal glands are innervated and exhibit functioning Muscarinic M_1 and M_3 receptors, roughly in a 1:2 ratio between the two types of receptors (Mak and Barnes, 1990). Muscarinic M_3 receptors probably mediate electrolyte and water secretion in combination with M_1 receptors, but muscarinic M_3 receptors are the primary receptor that mediates mucus production. Muscarinic M_3 receptors also mediate mucus secretion (Ramnarine *et al.*, 1996). In response to the stimulation of muscarinic receptors, goblet cells can also generate mucus, although this process requires relatively high concentrations of agonists (Rogers, 2001).

Mucus hypersecretion is a pathological hallmark of asthma and is significantly attributed to airflow limitation obstructing the airways (Jeffery, 2004). This feature can be seen in asthma. Acute airway inflammation can regulate mucus hypersecretion by increasing the acetylcholine released. This is possible because the production of mucus in the central airways is vagally mediated to a large extent. Additionally, stimulation of cholinergic receptors interacts synergistically with epidermal growth factor (EGF) to activate mucus cells in airway submucosal glands (Iwase *et al.*, 2002). A study by O'Donnell *et al.* (2004) showed that goblet cell hyperplasia and mucus gland hypertrophy in asthma are thought to be regulated by EGF. This finding may have additional implications for the effects of muscarinic receptors on this pathology. Stimulation of muscarinic receptors causes transactivation of the EGF receptor in conjunctival goblet cells, which is an essential component in mucin production by these cells (Kanno *et al.,* 2003). This opens up the actual prospect that increased endogenous acetylcholine release may encourage the remodeling of mucus-secreting cells in asthma, but further research on human subjects is required.

2.4.4 Airway remodeling

In chronic airway illnesses such as asthma, airway inflammation is frequently linked with cellular and structural changes in the airways, a process known as airway remodeling (Jeffery, 2001). Hubert and Koessler originally described airway remodeling in fatal asthma cases in 1992 (Redington and Howarth, 1997). Airway remodeling is a progressive component of permanent airflow limitation in many disorders (An *et al.*, 2007), and it corresponds with disease severity (Hogg *et al.*, 2004). Airway remodeling has been reported in all asthma severity stages and large and small airways (James *et al.*, 2002).

In asthma, mucus gland enlargement, goblet cell hyperplasia, and pulmonary vascular remodeling characterize airway remodeling (Jeffery, 2001). Airway remodeling refers to structural changes in large and small airways due to various disorders such as asthma. Subepithelial fibrosis, increased smooth muscle mass, gland enlargement, neovascularization, and epithelial abnormalities are all asthma-related changes. In addition, airway remodeling refers to structural alterations in the airways of asthmatic individuals that are not observed in healthy individuals. These structural changes

include loss of epithelial integrity, basement membrane thickening, subepithelial fibrosis, growth of goblet cells and submucosal glands, increased smooth muscle mass, decreased cartilage integrity, and increased airway vascularity (Bergeron, Tulic and Hamid, 2010a). These structural changes are thought to result from a continuing chronic inflammatory process involving the activation of inflammatory cells such as $CD4^+$ T cells, eosinophils, neutrophils, and mast cells (Kroegel *et al.*, 1994; Metcalfe *et al.*, 1997).

In addition, asthma is characterized by a thicker foundation membrane, subepithelial fibrosis, and significant thickening of the airway smooth muscle bundle (An *et al.*, 2007). Moreover, anatomical abnormalities in the airway may exacerbate lung function decline (Paré *et al.*, 1985). In severe asthma, mast cells invade smooth muscle and promote airway remodeling by releasing inflammatory mediators such as amphiregulin (Nakagome and Nagata, 2011). Although debatable, airway remodeling is typically linked to a chronic inflammatory process. These remodeling alterations contribute to airway wall thickening, which results in airway narrowing, bronchial hyperresponsiveness, airway edema, and mucous hypersecretion. In asthmatic patients, airway remodeling is related to poor clinical outcomes. The asthma period is connected with decreased lung function, increased AHR, asthma symptoms, and increased drug use.

2.5 β2-adrenergic receptor regulation of airway smooth muscle

β2- adrenergic receptors can mainly be found in airway smooth muscles. These receptors are also in skeletal muscles, cardiac muscles, uterine muscles, alveolar type II cells, mast cells, mucous glands, epithelial cells, vascular endothelium, eosinophils, and lymphocytes. β2-adrenergic receptors are transmembrane glycoprotein structures that induce an intracellular reaction in response to catecholamines. These receptors are members of a significant receptor family (R7G) encompassing receptors that respond to substances other than catecholamines, and these receptors are also associated with guanine nucleotide (GTP)-binding proteins (G proteins). The β2-adrenoceptor is a receptor that goes through the cell membrane and has at least three subtypes, including beta-1 (β1), beta-2 (β2), and beta-3 (β3). Alpha-1 and alpha-2 receptors are additional adrenergic receptors (Abosamak and Shahin, 2022).

A study has asserted that β1-adrenoceptors are primarily found in the heart, while β2-receptors are located in the lungs, liver, blood vessels, and uterus muscles. Inside the airways, most β2-adrenoceptors are found on the smooth muscle in the airways. They can also be found in type II pneumocytes, endothelial cells, and mast cells (Yusuf *et al.*, 2019). Activating the β2-adrenergic receptor in the human lung can lead to smooth muscle relaxation, inhibit the release of acetylcholine from cholinergic nerve terminals, stimulate mucus cell secretion, and prevent mediator release from inflammatory cells (Bai, 1992). β2-adrenoceptors work on the activation of G protein-coupled adenylate cyclase, increase cAMP, and prevent the release of Ca^{2+} from intracellular stores. This action causes smooth muscles to relax and bronchi to open up (bronchodilation) (Figure 2.5). Human airway smooth muscle includes just β2-receptors, which, when activated, cause an increase in intracellular cAMP and the activation of protein kinase A (Barnes, 1993). β-Adrenergic receptors are connected to Gs and can activate adenylate cyclase and elevate the cAMP level. cAMP raises protein kinase A (PKA) activity by phosphorylating downstream protein regulators. The activation of this signal transduction pathway can result in the suppression of phosphoinositol hydrolysis, a decrease of Ca^{2+} levels in the intracellular, and the activation of large conductance potassium channels, which is essential for the resulting relaxation. (Proskocil and Fryer, 2005).