

Invited Review

Eosinophils and airway nerves in asthma

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Summary. In the lungs, neuronal M₂ muscarinic receptors limit the release of acetylcholine from post-ganglionic cholinergic nerves. However, these receptors are not functional under certain circumstances in animal models of hyperreactivity such as occurs after exposure of sensitised animals to an allergen or during a respiratory tract virus infection. This loss of M₂ receptor function leads to an increase in acetylcholine release from cholinergic nerves and thus is a mechanism for the vagally mediated hyperreactivity seen in these animals. Studies in animal models of hyperreactivity have shown that eosinophils localise to the airway nerves of sensitised animals after antigen challenge. Inhibiting this localisation of eosinophils either with an antibody to the eosinophil survival cytokine IL-5 or the eosinophil adhesion molecule VLA-4 prevents loss of M₂ muscarinic receptor function. It is likely that eosinophil MBP is responsible for the loss of M₂ receptor function, since inhibiting eosinophil MBP with an antibody or neutralising MBP with heparin prevents this loss of function. These data are also supported by ligand binding studies where it has been shown that eosinophil MBP is an allosteric antagonist at neuronal M₂ muscarinic receptors. Loss of function of lung neuronal M₂ muscarinic receptors may also occur under certain circumstances in patients with asthma, although the mechanisms are not yet established.

Key words: Vagus nerves, Major basic protein, Hyperresponsiveness, Muscarinic receptors

Introduction

Asthma is a common clinical condition characterized by the symptoms of an intermittent cough, wheeze and breathlessness. Physiologically, asthma is characterised by a tendency for the airways to contract excessively

when exposed to a variety of compounds, this is termed hyperreactivity. The cause of the hyperreactivity is uncertain however, it may be due to increased activity of the parasympathetic nerves, the nerves that provide the innervation of the airway smooth muscle.

The typical pathological features of asthma include an inflammation of the airways with eosinophils and lymphocytes. Recent studies suggest that it is the infiltration of the airways by eosinophils which causes the hyperreactivity. One mechanism that links the overactivity of the parasympathetic nerves and inflammation by eosinophils has been the finding that eosinophils inhibit a particular group of autoreceptors on the parasympathetic nerves, the M₂ muscarinic receptors. In this review we will discuss the effect of eosinophils on parasympathetic nerve function in animal models of hyperreactivity and in humans with asthma.

The anatomy and physiology of the pulmonary parasympathetic nerves

The cell bodies of the vagus nerves lie in the nucleus ambiguus in the brain stem. From these cell bodies pre-ganglionic nerve fibers extend to parasympathetic ganglia which are interspersed along the posterior aspect of the trachea and bronchi (Honjin, 1956; Richardson, 1979). The post-ganglionic fibers originating from these ganglia innervate the airway smooth muscle, the bronchial circulation and the glandular acini (Spencer and Leof, 1964; El-Bermani and Grant, 1975; El-Bermani, 1978; Baker et al., 1986). Post ganglionic efferent fibers extend to the level of the terminal bronchi. The major bronchi are the site of densest parasympathetic innervation and this is also the site of bronchoconstriction in patients with asthma (Nadel et al., 1971; Richardson, 1979; ten Berge et al., 1996). Stimulation of the parasympathetic nerves releases acetylcholine which causes the airway smooth muscle to contract (Colebatch and Halmagyi, 1963; Olsen et al., 1965; Green and Widdicombe, 1966; Madison et al., 1987; Maeda et al., 1988), the glandular tissue to secrete mucus (Brody et al., 1972; Gallagher et al., 1976) and the bronchial circulation to dilate (Widdicombe, 1966;

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Phipps and Richardson, 1976). Thus, since stimulation of the parasympathetic nerves mimics the features of asthma and since the site of obstruction in asthma corresponds to the area most densely innervated by these nerves it is likely that the parasympathetic nerves play an important role in the pathogenesis of asthma.

Muscarinic receptor subtypes in the airways.

Five muscarinic receptor subtypes (M_1 - M_5) have been identified based on their different genetic sequences. These receptors can also be distinguished from each other because they have differing binding affinities for muscarinic antagonists. Muscarinic M_1 receptors are selectively blocked by pirenzepine, muscarinic M_2 receptors are blocked by AF-DX116 and gallamine, muscarinic M_3 receptors are blocked by 4-DAMP, while M_4 receptors are antagonized by himbicine. Selective antagonists at muscarinic M_5 receptors have not yet been identified.

In the lungs, studies have demonstrated that muscarinic M_1 and M_2 receptors are located along nerve bundles and within the cholinergic ganglia, post ganglionic cholinergic nerves possess only M_2 muscarinic receptors (Fryer et al., 1996). Airway smooth muscle cells express M_2 and M_3 muscarinic receptors (Madison et al., 1987; Maeda et al., 1988). Airway mucous glands express M_1 and M_3 muscarinic receptors and these receptors are also located in the periphery of the lung, where the function is uncertain.

Vagally induced bronchoconstriction is limited by neuronal muscarinic M_2 receptors

Stimulation of the parasympathetic nerves leads to contraction of the airway smooth muscle. Increased activity of the vagus nerves causes excessive narrowing of the airways. Since increased activity of the parasympathetic nerves can have such important consequences the activity of these nerves needs to be controlled. Control of acetylcholine release from the parasympathetic nerves is exerted by acetylcholine itself. Acetylcholine acts on muscarinic M_2 autoreceptors located on post-ganglionic nerves, stimulation of these receptors limits further acetylcholine release (Fig. 1), (Fryer and Maclagan, 1984; Blaber et al., 1985; Faulkner et al., 1986). Thus, M_2 muscarinic receptors act as autoreceptors and their function is to limit vagally-induced bronchoconstriction.

The function of the neuronal M_2 autoreceptor was first demonstrated, *in vivo*, by demonstrating that vagally-induced bronchoconstriction was modified by drugs that act on M_2 muscarinic receptors. For example, the M_2 muscarinic receptor antagonist gallamine causes a dose dependent potentiation of vagally-mediated bronchoconstriction (Fryer and Maclagan, 1984). Conversely, administration of pilocarpine which stimulates M_2 muscarinic receptors decreases vagally-induced bronchoconstriction. The presence of functional

M_2 receptors has also been demonstrated, *in vitro*, by directly measuring changes in induced acetylcholine release using high performance liquid chromatography in the presence of selective M_2 receptor antagonists (Patel et al., 1995). Although first described in the airways of guinea pigs, functional M_2 muscarinic receptors have since been described in the airways of all species studied, including, in humans (Minette and Barnes, 1988; Patel et al., 1995).

Loss of function of neuronal muscarinic M_2 receptors causes vagally-mediated hyperreactivity

Antigen challenge of sensitized animals causes an immediate temporary bronchoconstriction that is followed by a prolonged period of increased reactivity to a variety of compounds such as methacholine or histamine. This second, long-term period of hyperreactivity, can be completely inhibited by blockade of the parasympathetic nerves, indicating that antigen-induced hyperreactivity is vagally-mediated (Santing et al., 1995; Costello et al., 1997). This is supported by the finding that there is increased concentrations of acetylcholine in the airways of antigen-challenged mice (Larsen et al., 1994) and dogs (Walters et al., 1986). These data suggest that antigen-induced hyperreactivity is due to increased release of acetylcholine from the vagus nerves.

Under normal circumstances neuronal M_2 muscarinic receptors limit acetylcholine release from the vagus nerves but since it has been reported that there is increased release of acetylcholine in antigen-challenged animals this suggests that M_2 muscarinic receptors may not be functional in antigen-challenged animals. Hence

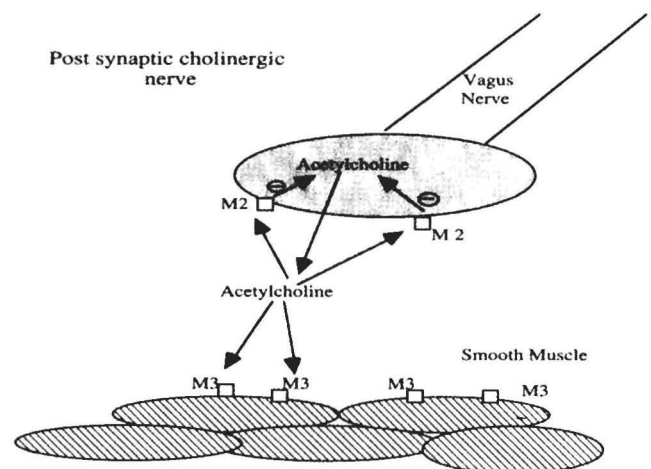


Fig. 1. The pulmonary neuronal M_2 muscarinic receptor limits acetylcholine release from parasympathetic nerves. Acetylcholine released from pulmonary vagal nerves stimulates muscarinic M_3 receptors on airway smooth muscle causing smooth muscle contraction, at the same time acetylcholine stimulates M_2 muscarinic receptors located on the post-ganglionic nerves. Stimulation of these neuronal M_2 muscarinic receptors limits further acetylcholine release.

studies to investigate the function of M_2 muscarinic receptors were performed in antigen-challenged guinea pigs. In antigen sensitised animals studied 24 hours after antigen challenge it was shown that, in contrast to control animals, gallamine did not potentiate and pilocarpine did not attenuate the magnitude of vagally-induced bronchoconstriction (Fig. 2). These data indicate that there is loss of function of neuronal M_2 muscarinic receptors in antigen-challenged animals. It was also shown that M_3 receptors on the airway smooth muscle were functional since the bronchoconstriction induced by acetylcholine was the same in both control and antigen challenged animals, when the vagus nerves were inhibited by vagotomy (Fryer and Wills-Karp, 1991). Dysfunction of neuronal M_2 muscarinic receptors has been confirmed in other experiments using different

models of antigen challenge and in different species (Fryer and Jacoby, 1992; ten Berge et al., 1995; Costello et al., 1997; Evans et al., 1997; Fryer et al., 1997; Belmonte et al., 1998).

Mechanisms of loss of function of neuronal M_2 receptors in antigen challenged animals

Studies in experimental animals have established that eosinophils play a pivotal role in the pathogenesis of antigen-induced hyperreactivity (Pretolani et al., 1994; Lefort et al., 1996). Since antigen-induced hyperreactivity is caused by loss of function of M_2 receptors the role of eosinophils in this dysfunction has been investigated.

The role of eosinophils in loss of function of M_2

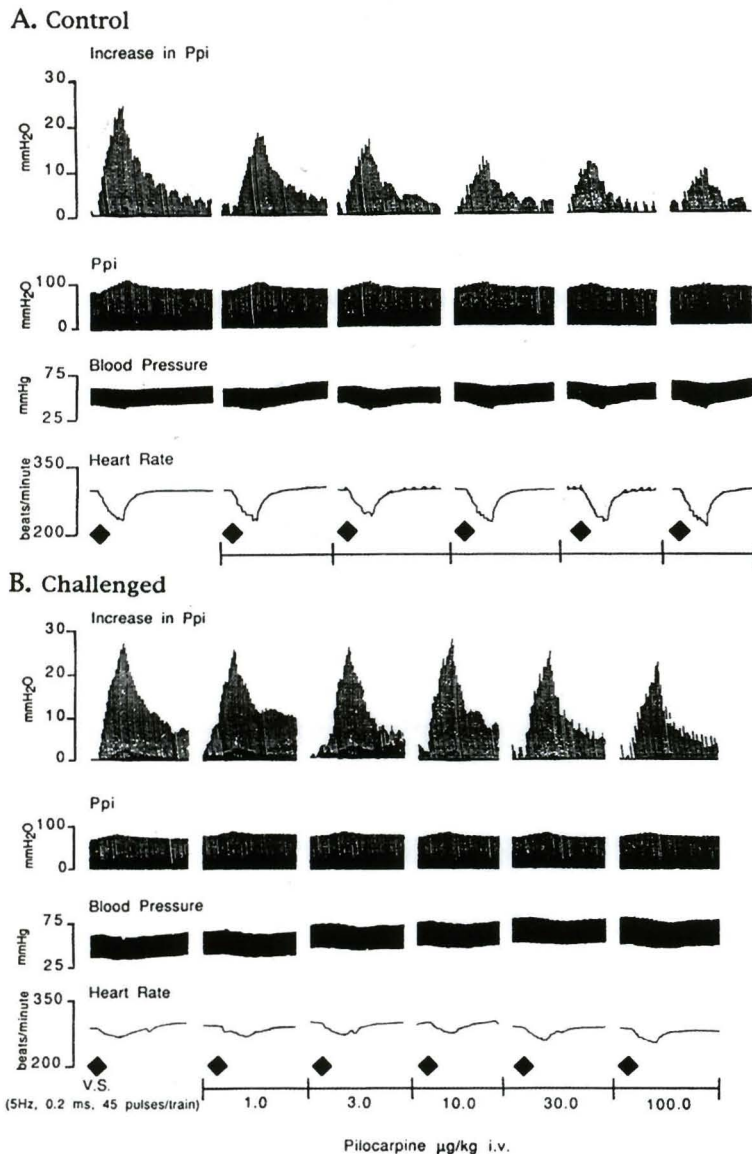


Fig. 2. Normal function of neuronal M_2 muscarinic receptors is seen in control (A) but not in antigen challenged guinea pigs (B). The figure shows that cardiac and respiratory responses to intermittent vagal nerves stimulation (shown by diamonds) in an anaesthetized, paralyzed and ventilated guinea pigs. The bronchoconstrictor responses to vagal nerve stimulation are shown as a change in pulmonary inflation pressure (magnified tenfold, in the second panel). The heart rate and blood pressure recordings are shown in the lower two panels. There is a dose dependent inhibition of the magnitude of vagally induced bronchoconstriction following the administration of the M_2 muscarinic receptor agonist pilocarpine in control animals (A). However pilocarpine does not inhibit vagally induced bronchoconstriction in antigen challenged animals (B).

receptors in antigen challenged animals has been demonstrated. This was shown by pretreating antigen sensitised guinea pigs with an antibody to the eosinophil growth factor, interleukin-5, which selectively depleted circulating eosinophils, before antigen challenge. In these studies pretreatment with the antibody was shown to prevent antigen-induced loss of function of neuronal M_2 muscarinic receptors (Elbon et al., 1995). In further studies it was shown that pretreatment with an antibody to the eosinophil adhesion molecule VLA-4 prevented antigen-induced eosinophil accumulation in the airways and also prevented both the loss of function of M_2 muscarinic receptors and the development of airway hyperreactivity (Fryer et al., 1997). The results of these studies suggested that eosinophils are responsible for the loss of pulmonary neuronal M_2 muscarinic receptor function in the airways of antigen-challenged guinea pigs.

Eosinophil cationic proteins mediate antigen-induced loss of function of M_2 muscarinic receptors

M_2 muscarinic receptors contain a core of sialated glycoproteins, which give the receptor a negative charge. Many antagonists at M_2 muscarinic receptors are positively charged and it has been speculated that this cationic charge is important for antagonist binding to M_2 receptors (Hu et al., 1992). The cytoplasm of eosinophils contain electron dense granules which store cationic proteins. Four main cationic proteins have been identified; eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN), eosinophil peroxidase (EPO) and eosinophil major basic protein (MBP) (Gleich and

Loegering, 1973; Gleich et al., 1976). These granular proteins possess high isoelectric points (pH 10 to 11.5) and are toxic to mammalian cells.

The cationic nature of these proteins prompted an investigation to assess if these cationic proteins might also be antagonists at M_2 receptors. In receptor binding studies on M_2 and M_3 muscarinic receptors MBP was found to displace the muscarinic antagonist [3 H]N-methylscopolamine ([3 H]NMS) from guinea pig and human M_2 , but not M_3 , muscarinic receptors (Jacoby et al., 1993, 1995). Thus indicating that MBP is an antagonist at M_2 but not M_3 receptors. Further studies showed that MBP was an allosteric rather than competitive antagonist at M_2 receptors. Additional studies showed that the anionic compound heparin could displace MBP from these receptors, suggesting that the antagonism of MBP for these receptors was reversible (Fryer et al., 1992). Eosinophil MBP may have a physiologically relevant role in the loss of M_2 receptor function, since the dissociation constant for MBP at M_2 receptors is 1.4×10^{-5} M, which is similar to the concentration of MBP found in the sputum of patients with acute asthma (Frigas et al., 1981).

In order to establish a role for eosinophil MBP in the loss of M_2 receptor function, *in vivo* studies were performed with a specific neutralizing antibody to eosinophil MBP (Evans et al., 1997). Pretreatment of antigen sensitized guinea pigs with the antibody to MBP before challenge protected the function of M_2 receptors and prevented antigen-induced hyperreactivity. In other studies antigen-challenged animals were administered the anionic neutralizing compounds heparin or poly L-lysine both of which acutely restored M_2 receptor

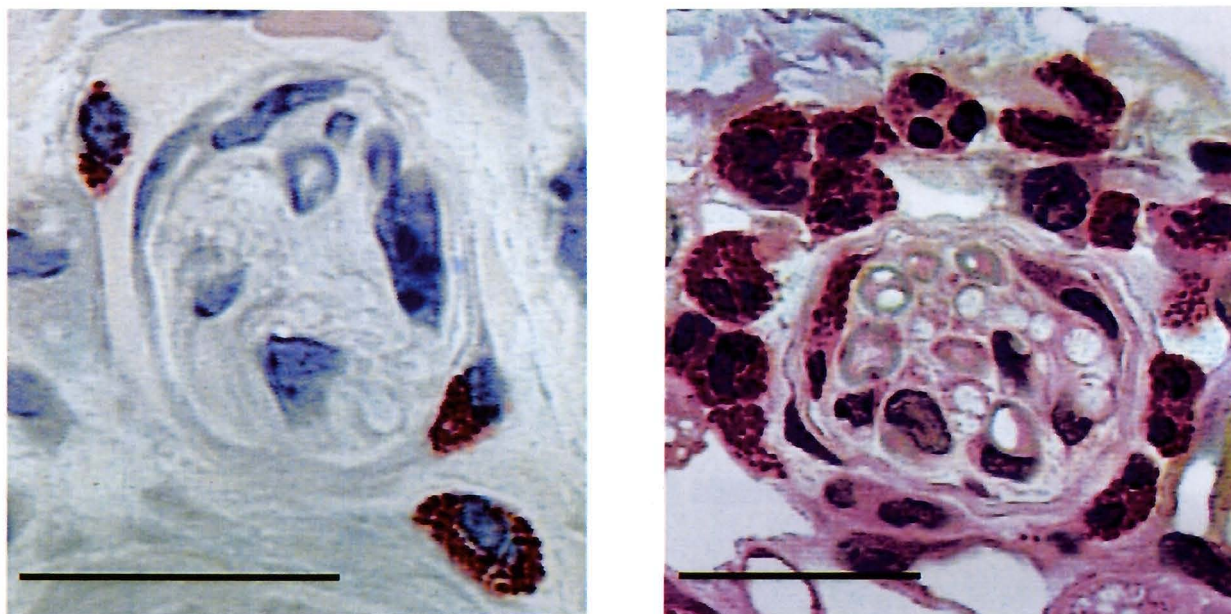


Fig. 3. Eosinophils are found in association with nerve bundles in the airways of antigen challenged guinea pigs. The photomicrographs are from methacrylate embedded sections of antigen challenged guinea pig bronchus. Eosinophils have been detected with Luna's stain (right) and haematoxylin and eosin (left). Eosinophils are seen surrounding and also located inside the epineurium of the nerve bundles. Eosinophils were only rarely seen in association with airway nerves in control non-challenged animals. Bar: 20 μ m. (Reproduced from Costello et al., 1997).

function in antigen-challenged guinea pigs and rats (Fryer and Jacoby, 1992; Belmonte et al., 1998) has been investigated. These findings suggest that neuronal M₂ muscarinic receptors become dysfunctional after antigen challenge because eosinophil MBP is acting as an endogenous antagonist at neuronal M₂ receptors.

Eosinophils are anatomically associated with airway nerves in antigen-challenged guinea pigs

Eosinophil MBP is highly cationic and does not diffuse within the tissues after it is released from eosinophils, thus sections of airways of antigen-challenged animals were studied to determine whether eosinophils, which store MBP, localize to and degranulate on airway nerves. In these studies airway nerves were identified using immunohistochemical techniques and a close anatomical association of eosinophils and nerves was observed. In antigen-challenged guinea pigs eosinophils were seen in greater numbers closely associated with airway nerve fibers in the submucosa and the smooth muscle of antigen-challenged animals compared to control animals (Fig. 3). Both the numbers and the proportion of eosinophils associated with airway nerves were higher in antigen challenged animals compared to control non-challenged animals, suggesting that after antigen challenge there may be a process that results in the localization of eosinophils to the airway nerves. Compared to control animals significantly more eosinophils were seen in close association (<15 μ m) with airway cholinergic ganglia and along airway nerve bundles in antigen-challenged animals. The number of eosinophils per nerve was inversely correlated with the *in vivo* function of the neuronal M₂ muscarinic receptor, in other words the greater the number of eosinophils the less functional the M₂ muscarinic receptor. These data suggest that eosinophils are important in the loss of function of M₂

muscarinic receptors (Costello et al., 1997).

A greater number of eosinophils (per mm²) was seen around the airway nerve bundles than was seen either within the whole airway wall or around the adjacent blood vessels, suggesting that there may be a mechanism whereby eosinophils are actively recruited to airway nerves (Fig. 4). Thus, there is good evidence to indicate that eosinophils are involved in the loss of function of neuronal M₂ muscarinic receptors in antigen-challenged animals.

Increased cholinergic activity in the airways of patients with asthma

Compared to control non-asthmatics administration of inhaled anticholinergic agents causes significantly less bronchodilation than in subjects with asthma with similar resting pulmonary function tests, indicating that there is increased resting vagal airway tone is higher in patients with asthma (Molfino et al., 1993). In addition, some patients with asthma demonstrate increased vagal hyperreactivity. Thus there is increased cholinergic activity in asthma.

Function of neuronal M₂ muscarinic receptors in patients with asthma

In vitro tests on the function of the neuronal M₂ muscarinic receptor in patients with asthma have not yet been performed since bronchial tissue is rarely resected from patients with asthma. *In vivo* investigations on the function of the M₂ receptor in patients with asthma have been hampered because it is not possible to directly stimulate the vagus nerves *in vivo*. Thus, *in vivo*, studies have relied on techniques that indirectly stimulate a vagally-mediated bronchoconstriction. In these studies vagally-mediated bronchoconstriction has been induced by having subjects inhale sulphur dioxide (Minette et al.,

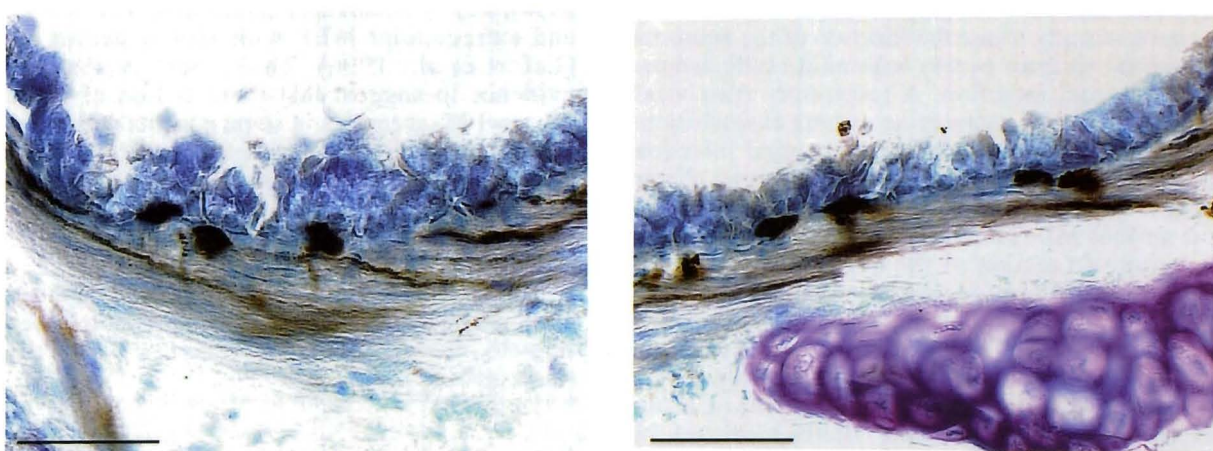


Fig. 4. Photomicrograph shows the localization of eosinophils to airway nerve fibres in the smooth muscle of antigen challenged guinea pigs. Airway nerve fibers, from two separate animals, were identified using acetylcholinesterase immunohistochemistry, eosinophils were identified by peroxidase staining. Eosinophils were seen in greater density in association with airway nerve fibres than in control animals. Bar: 50 μ m. (Reproduced from Costello et al., 1997).

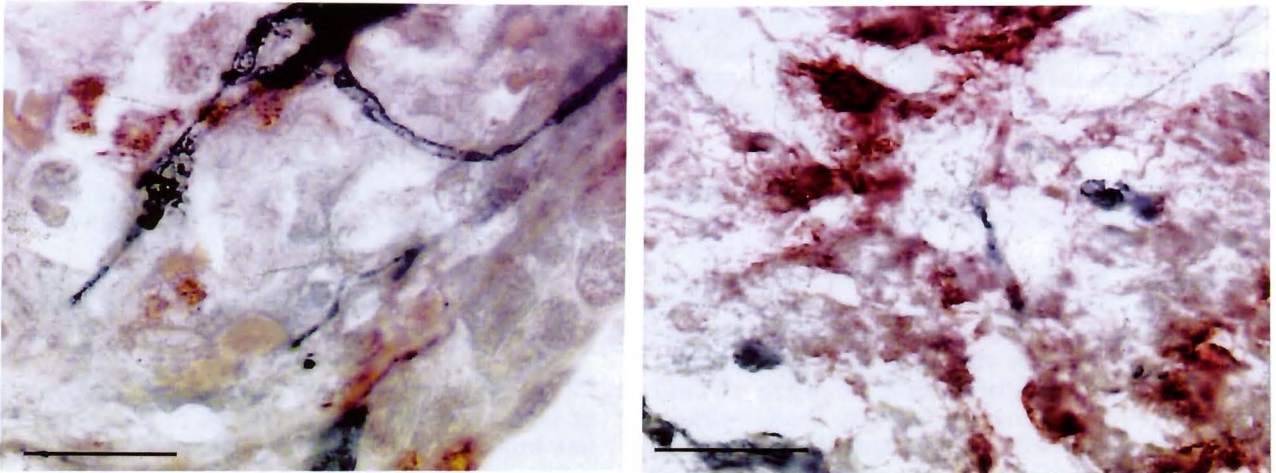


Fig. 5. Eosinophils and extracellular MBP are found in association with nerve fibers in the airways of asthmatics who died during an asthmatic attack. The photomicrograph is from a paraffin embedded section of an asthmatic's airway, eosinophils and extracellular MBP were detected with an antibody to human MBP, which is shown in red. The photomicrograph on the left shows intact eosinophils some of which are undergoing degranulation in close proximity to the cell body of an airway nerve fiber (detected with an antibody to PGP 9.5, in black). The photomicrograph on the right shows sheets of extracellular MBP is seen in close association with small nerve fibers. Bar: 20 μm . (Reproduced from Costello et al., 1997).

1989), histamine (Ayala and Ahmed, 1989), a beta receptor antagonist (Okayama et al., 1994) or cold air (Hurst et al., 1998) all of which indirectly stimulate a vagal reflex bronchoconstriction. In these studies the ability of the muscarinic receptor agonist pilocarpine to modify the induced bronchoconstriction has then been investigated. In two of these studies subjects with asthma were shown to have dysfunctional neuronal M_2 receptors (Ayala and Ahmed, 1989; Minette et al., 1989), while in the other studies normal M_2 receptor function was demonstrated (Okayama et al., 1994; Hurst et al., 1998). The reasons for the differences in the results of these studies may reflect the differences in the techniques used to induce the vagal reflex bronchoconstriction or alternatively may reflect the severity of the asthma in the subjects studied.

We have recently tested the function of the neuronal M_2 muscarinic receptor people with mild stable asthma and during a viral infection. A respiratory tract viral infection is a common cause of an asthma exacerbation. In guinea pigs it has been shown that a viral infection causes loss of function of neuronal M_2 receptors (Fryer and Jacoby, 1991). In subjects with asthma the studies showed normal M_2 receptor function at baseline but transient loss of function of the M_2 receptor during a viral respiratory infection (Keen et al., 1998). This observation of heightened cholinergic activity during a viral infection may help explain the previous observations that have shown that anticholinergic agents are particularly effective in the management of acute asthma attacks, which are usually virally mediated. In summary, studies on the function of the neuronal M_2 receptor in humans with asthma indicate that there may be some individuals who have persistent loss of M_2 receptor function and others who develop loss of

function during periods of instability.

The underlying mechanisms of loss of function of M_2 receptors in patients with asthma have not been investigated. However some similarities between the findings in animal studies and those from the human studies with have been found. For example, eosinophil major basic protein has been shown to be an allosteric antagonist at human M_2 muscarinic receptors (Olsen et al., 1965). Further similarities with animal models of hyperreactivity include the finding that in patients with asthma that there is a close anatomical relationship of eosinophils and airway nerves. Using a double immunohistochemical staining technique to detect eosinophil MBP and neural tissue in airway sections from patients who died during an asthma attack we have shown a close anatomical association of both eosinophils and extracellular MBP with airway nerves (Fig. 5), (Lefort et al., 1996). Thus, there is some indirect evidence to suggest that there is loss of function of neuronal M_2 receptors in some patients with asthma and that eosinophil MBP may play a role in this loss of function. Further studies are required to investigate the role of eosinophils in the loss of M_2 receptor function in humans with asthma.

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