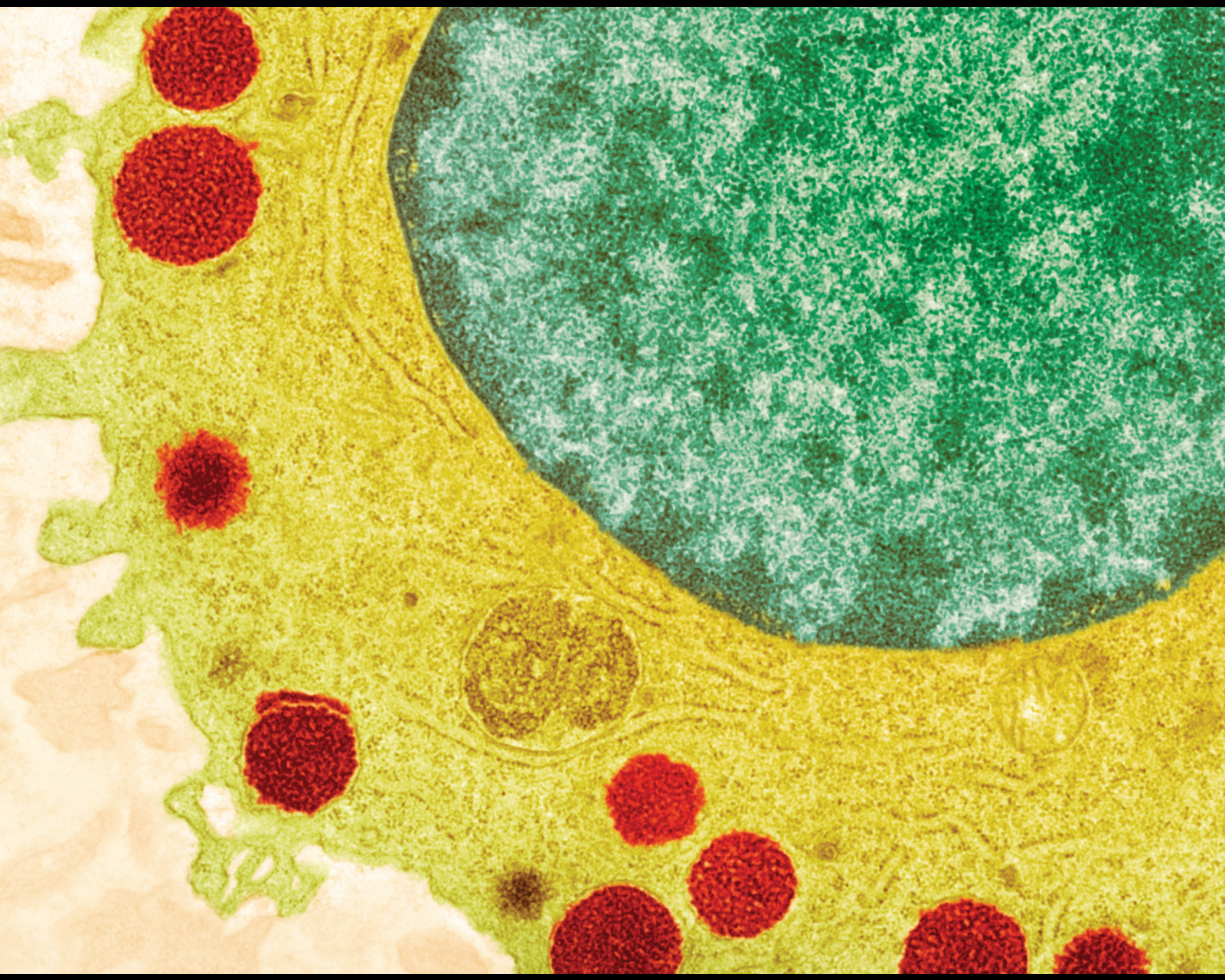
Journal of Allergy

Smooth Muscle Hypercontractility in Airway Hyperresponsiveness: Innate, Acquired, or Nonexistent?

Guest Editors: Ynuk Bossé, Éric Rousseau, Yassine Amrani, and Michael M. Grunstein



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Editorial Board

Contents

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Editorial

Smooth Muscle Hypercontractility in Airway Hyperresponsiveness: Innate, Acquired, or Nonexistent?

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1. Introduction

Asthma symptoms are triggered or exacerbated by a range of environmental factors, such as allergens, viruses, fungi, exercise, aspirin, pollutants, and occupational irritants and sensitizers. While traditionally considering an intrinsic disease, in more recent years asthma has been viewed by many as a genetically associated environmental lung disorder with a heterogeneous pathogenesis. With the exception of the severe cases, the diagnostic signature of asthma is the reversibility of airway obstruction by agents that relax airway smooth muscle (ASM), which attests to the importance of this tissue in the pathobiology of the airflow obstruction.

Most asthmatic individuals are hyperresponsive to bronchoprovocative challenge with a spasmogen (i.e., bronchoconstrictor agonist such as methacholine or histamine). Airway hyperresponsiveness (AHR) in asthmatic patients can either result from "hyperreactive airways" characterized by an excessive airway narrowing or "hyperexcitability," where the airways become excessively sensitive to very low doses of constrictor agonists. It is believed that the abnormal narrowing of the airways (hyperreactivity) is responsible for most of the morbidity and mortality due to asthma. In either case, the role of ASM in the development of AHR remains to be further investigated. The controversial questions that remain to be answered are whether AHR seen in asthmatic patients is due to functional changes in the ASM and whether those changes actually lead a "hypercontractile" phenotype. This special issue aims to shed light on what seems to be a perdurable debate as to whether the hypercontractility of ASM characterizes AHR, whether this hypercontractile phenotype exists, and whether it is innate or acquired. Notwithstanding the potentially important associative role of airway inflammation, this special issue addresses different viewpoints by experts in the field that relate to newly identified contractile properties of ASM that may contribute to AHR when perturbed and also considers the latest advances in the search for better asthma treatments that directly target the ASM.

C. D. Pascoe and coworkers set the stage for the ongoing debate by providing an enlighten historical perspective on the role that has been attributed to ASM in the pathobiology of asthma and AHR. The authors reference monographs that date backs to the 16th century and then describe pivotal developments made more recently that offer tentative links between the airway dysfunction seen in asthma and certain recognized features of ASM observed *ex vivo*. They also point out the rapid gain of interest and the increasing amount of research pursued in this area in the past few years.

2. Innate Hypercontractility

The review by R. Berair and coworkers consider the role that intrinsic abnormalities of asthmatic ASM play in the manifestation of AHR. They discuss the accumulation of evidence that demonstrates that asthmatic ASM cells shorten more and quicker when studied either in isolation or embedded in collagen gels. These authors also describe the molecular mechanisms that have been proposed as responsible for the hypercontractile phenotype. However, as acknowledged by the authors, it is difficult to determine if those abnormalities are innate or acquired. Genetic differences can certainly be involved, but certain epigenetic changes that may be acquired *in vivo* and that are preserved in cell/tissue culture are other important considerations. In the latter case, the muscle may not be inherently abnormal but, rather, may be rendered hypercontractile because it had been previously exposed and operated in an altered microenvironment.

3. Acquired Hypercontractility

Within the context that AHR in asthmatics may be due to acquired ASM hypercontractility, a multitude of asthma triggers and ensuing inflammatory/immunologic mediators have been shown to modify ASM function. Accordingly, ASM contractility is not viewed as static (fixed) in nature but, rather, to be plastic (adaptable). This is consistent with *in vivo* observations showing that the degree of airway responsiveness is variable in time and in response to different interventions [1]. Such plasticity of ASM may allow a "normal" ASM to become hypercontractile and thereby contribute to AHR.

In the article by L. Xiao and Z. X. Wu, these authors demonstrate that prolonged exposure of mice to side-stream tobacco smoke *in vivo* increases the force generated by tracheal rings exposed *ex vivo* to both substance P and electrical field stimulation. This is a good example of how an inhaled environmental factor can contribute to AHR by increasing ASM contractility.

Many endogenous mediators that are overexpressed in asthma, such as cytokines, enzymes, lipids, and adhesion molecules, can also alter ASM contractility (reviewed recently in [2]). New intracellular lipid signaling molecules have also been identified recently [3] for their specific role in regulating Ca^{2+} sensitivity. The regulation of their expression through the activation of nuclear factors is now under scrutiny. Hence, not only proteomics and transcriptomics but also metabolomics are likely to shed lights on experimental and clinical AHR.

Alterations in the structural microenvironment in which ASM is embedded may also contribute to a multitude of defects leading to the development of asthma symptoms and AHR. For example, extracellular matrix (ECM) components may affect ASM responsiveness to both spasmogens and bronchodilators. In this special issue, C. M. Teoh and coworkers discuss signaling crosstalks that have been identified between ECM-binding integrins and G protein-coupled receptors (GPCR) in cells derived from different tissues. Since several integrins are expressed on ASM and many of which, as well as their cognate ligands, are dysregulated in asthma, the authors raised the possibility that similar crosstalks may exist in asthmatic ASM that contribute to both AHR and/or hyporesponsiveness to bronchodilators.

Further contributing to the concept of altered signaling in asthmatic ASM, J. A. Jude and coworkers report herein that the induction of CD38, a multifunctional enzyme that catalyzes the synthesis and hydrolysis of cyclic ADP-ribose (cADPr) from NAD(+) to ADP-ribose, by inflammatory cytokines, such as IL-13 and TNFα, is enhanced in asthmatic compared to nonasthmatic ASM cells. In agreement with recent studies [4], abnormal CD38 pathways may enhance ASM contractility, via the release of Ca^{2+} from the sarcoplasmic reticulum by cADPr acting on ryanodine receptors. This evidence argues that the enhanced contractility would be acquired because of the exposure to inflammatory cytokines, and the contribution of this molecular pathway in the development of asthmatic AHR would reflect the intrinsic difference of asthmatic ASM cells that fosters a greater CD38 upregulation in response to cytokine exposure. This finding supports current growing evidence describing phenotypic changes in ASM between healthy and asthmatic subjects [5]. Accordingly, J. A. Jude and coworkers suggest that altered signaling mechanisms affecting the transcriptional control of CD38 expression or posttranscriptional mechanisms regulating CD38 RNA stability, such as microRNAs and RNA-binding proteins, may explain this enhanced induction of CD38 expression by cytokines in asthmatic ASM.

4. Nonexistent ASM Hypercontractility

AHR in asthmatics may also occur in the absence of intrinsic ASM hypercontractility, reflecting changes in asthmatic lungs that result from defects in nonmuscle factors. In this context, B. J. Canning and coworkers highlight the often understated role of the peripheral nervous system in asthmatic AHR. They suggest that the proven efficacy of anticholinergic drugs in attenuating airway responsiveness to direct and indirect bronchospastic stimulation testifies that an imbalance of the parasympathetic cholinergic and noncholinergic control of ASM activation is at the origin of AHR in asthma. They also point out that neuronal control of vascular tone is of utmost importance in determining the degree of airway responsiveness, since it affects the rate of clearance of spasmogens acting on ASM.

5. ASM Contractile Properties: Beyond Force and Ability to Relax

Earlier studies focused primarily on two particular ASM contractile properties, namely, the ability to generate stress (i.e., force/cross-sectional area) and the ability to relax either spontaneously or in response to bronchodilators. More recently, substantial research has been directed at understanding the role of ASM stiffness and the ability to tolerate and recover from oscillating stress. This stems from the realization that breathing maneuvers dynamically stretch the ASM *in vivo* and that length oscillations greatly affect ASM contractility [6, 7]. In their article, P. B. Noble and coworkers address this

topic by discussing the interlay that exists between airway wall stress versus strain in the absence and in the presence of airway wall stiffening induced by ASM activation and the impact of this interplay on ASM contractility and airway responsiveness. From *ex vivo* experiments using ASM strips or isolated airways to *in vivo* lung function measurements and asthmatic's "everyday" life symptoms, P. B. Noble and coworkers bring together the arguments in favor and against the claim that AHR in asthmatics is due to an impaired bronchodilatory response to breathing maneuvers.

The rate of airway renarrowing following the bronchodilatory effect of a deep inspiration also affects the magnitude of the relief obtained by such a maneuver. G. Ijpma and coworkers begin their review by pointing out seminal work that demonstrates that the rate of renarrowing is faster in asthmatic than in nonasthmatic individuals. The authors argue that this increased rate of renarrowing may be an *in vivo* reflection that one of the contractile properties of ASM that may be altered in asthmatics is the velocity of shortening. They then describe the growing body of data derived from animal models of asthma, human ASM cells and tissues, and computational models that suggest that quicker ASM shortening might be an underlying defect contributing to asthmatic AHR. The molecular mechanisms that may govern this increased velocity of shortening are also briefly discussed.

6. Current and Auspicious Treatments to Relief Airway Obstruction Caused by ASM

Independent of whether or not there are innate or acquired defects in asthmatic ASM that contribute to airway narrowing, the inhibition of ASM shortening is conducive for the treatment of asthma symptoms. In this regard, E. A. Townsend and coworkers highlight the strength and limitation of conventional bronchodilators and discuss four new classes of promising ASM relaxants including phosphodiesterase inhibitors, bitter taste receptor agonists, chloride channel modulators, and phytotherapeutics. Surprisingly, many of these drugs demonstrate bronchodilating potential despite elevating intracellular Ca^{2+}, suggesting that a very fine and localized balance of ions is required for proper ASM contraction and that interventions altering this balance may lead to therapeutic benefits.

Alternatively, why do not we just "get rid" of the perturbed asthmatic ASM? In his article, L. J. Janssen discusses the interventional approach called bronchial thermoplasty that functionally eliminates the ASM. This intervention involves delivering radiofrequency energy into the airways by means of an electrode introduced via a bronchoscope. The heat attained when the radiofrequency energy strikes the airway wall inhibits ASM contractility almost instantaneously, inducing apoptosis a few hours later, and the muscle mass then wanes over time. Despite the fact that only a small proportion of the airways is reachable by the bronchoscope, bronchial thermoplasty has been reported to improve symptoms, quality of life, and lung function in patients with varying asthma severity. According to L. J. Janssen, these early results may represent a foundation for the development of future less invasive strategies of ablating the ASM not only to alleviate symptoms temporarily but to ultimately "cure" asthma.

7. Conclusion

Although ASM does not seem to fulfill any important physiological function *per se*, its undisputed role in the pathophysiology of asthma warrants continued pursuit of research to understand by which mechanisms ASM activation induces AHR in asthmatics. Notably, the fact that inhibition of ASM shortening is salutary in the treatment of asthma symptoms does not necessarily imply that ASM is abnormal and the direct cause of AHR, as normal ASM responsiveness may also cause AHR when other lung defects are present. AHR can also be the consequence of heightened ASM activation due to either increased expression of inflammation-derived spasmogens, vagal dysregulation, or airway vascular abnormalities that either facilitate access of spasmogens to the ASM or alter their clearance. On the other hand, the existence of altered intrinsic ASM contractility has been reported in asthmatic ASM cells and tissues, and it will be important to determine whether this feature is innate or acquired *in vivo* and persists in culture because of epigenetic phenomena. Normal ASM can also be rendered hypercontractile because it operates in a "sick" environment. Hypercontractility does not only signify that ASM is stronger or has an attenuated ability to relax. It may also mean that it can shorten more or faster, or it can be stiffer or has a greater ability to tolerate or recover from oscillating stress that occurs *in vivo* due to natural breathing. Alterations in any of those contractile functions may contribute to AHR. Thus, more studies are clearly needed to understand the role of ASM in health and lung disorders.

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Review Article

Airway Smooth Muscle Hypercontractility in Asthma

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In recent years, asthma has been defined primarily as an inflammatory disorder with emphasis on inflammation being the principle underlying pathophysiological characteristic driving airway obstruction and remodelling. Morphological abnormalities of asthmatic airway smooth muscle (ASM), the primary structure responsible for airway obstruction seen in asthma, have long been described, but surprisingly, until recently, relatively small number of studies investigated whether asthmatic ASM was also fundamentally different in its functional properties. Evidence from recent studies done on single ASM cells and on ASM-impregnated gel cultures have shown that asthmatic ASM is intrinsically hypercontractile. Several elements of the ASM contraction apparatus in asthmatics and in animal models of asthma have been found to be different from nonasthmatics. These differences include some regulatory contractile proteins and also some components of both the calcium-dependent and calcium-independent contraction signalling pathways. Furthermore, oxidative stress was also found to be heightened in asthmatic ASM and contributes to hypercontractility. Understanding the abnormalities and mechanisms driving asthmatic ASM hypercontractility provides a great potential for the development of new targeted drugs, other than the conventional current anti-inflammatory and bronchodilator therapies, to address the desperate unmet need especially in patients with severe and persistent asthma.

1. Introduction

Asthma is a chronic inflammatory disease characterized by variable airflow obstruction and bronchial hyperreactivity associated with airway remodelling [1]. Most of asthma symptoms result from airflow obstruction caused by airway lumen narrowing. Although this narrowing is multifactorial in origin, abnormalities of airway smooth muscle (ASM) structure and function have been identified as one of the main causes [2]. Increased ASM mass has long been recognized as a major component of airway remodelling [3, 4]. More recently, asthmatic ASM was also found to be abnormal in its functional properties with increasing evidence showing intrinsic heightened contractility independent of other structural cells and independent of the asthma inflammatory milieu. In this paper we will examine the evidence of ASM hypercontractility in asthmatics, explore the potential mechanisms driving it, discuss its relevance, and briefly suggest its role in future asthma therapy.

2. Evidence of ASM Hypercontractility in Asthmatics

Abnormalities of asthmatic ASM structure and morphology have been described by Huber and Koesser more than 90 years ago when they reported increased ASM mass in a small group of patients who died of status asthmaticus compared to ASM form patients who died from nonpulmonary conditions [3]. This structural association has since been extensively described [4] although whether asthmatic ASM is also abnormal in function and if so whether this abnormality is an inherent property or only a result of the asthma inflammatory milieu has long been an unresolved question [5]. A few *in vitro* studies from the 1980–90s have tried to address this issue but the results have largely been conflicting. Compared to nonasthmatic controls, some studies suggested increased force generation in asthmatics ASM preparations; others showed no difference and even some seem to suggest decreased force generation in asthmatics [6–13]. Most of these studies had major methodological and statistical limitations

such as small sample size, failure to measure force per cross-sectional area (stress), failure to measure ASM shortening, and failure to identify ideal lengths for maximal contraction [14]. Furthermore, none of these studies examined ASM contractility at a cellular level, thus the mechanical effect of the extracellular nonmuscular connective tissue, and the biological effect of inflammatory cells and cytokines present in ASM preparations, on the final results could not be determined.

The first robust evidence of ASM hypercontractility in asthmatics was reported by Ma et al. This was the first study attempting to assess contractility characteristics of asthmatic ASM at cell level [15]. The study included 5 asthmatics and a similar number of nonasthmatic controls. 10–20 ASM cells were isolated from endobronchial biopsies collected from each subject. Maximum capacity and velocity of shortening of zero loaded single ASM cells in response to electrical field stimulation were measured under inverted phase-contrast microscopy. Asthmatics ASM cells showed significantly increased maximum capacity and velocity of shortening compared to controls. Although in this study the maximum shortening capacity in asthmatic ASM cells was increased by almost a third compared to controls, it should be considered that this shortening was measured at zero load and ASM cells *in vivo* would very likely shorten by a much lesser degree. This observation is pivotal but needs to be interpreted with caution due to the small sample size of this study.

Matsumoto et al. assessed asthmatic ASM contractility using a collagen gel contraction assay [16]. Gel percentage contraction to histamine was measured using floating gels containing ASM from 8 subjects with asthma and 9 nonasthmatic controls. These ASM containing gels were incubated overnight using 2 methods: attached or unattached to casting plates. The study found, using both methods, that histamine-exposed gels containing asthmatic ASM contracted more significantly.

More recently, Sutcliffe et al. also used gel contraction assay to assess ASM contractility in a much larger sample of 19 asthmatics and 8 healthy controls [17]. Gel contraction was measured every 15 minutes after stimulation with bradykinin. Results again showed significantly increased agonist-induced contraction in the asthma group (Figure 1).

Importantly, phenotypic plasticity of structural cells in culture cannot be completely excluded from studies done on primary ASM cultures, but we think this, if present, was minimal. The above evidence, in our opinion, confirms that asthmatic ASM is fundamentally different and hypercontractile and that this hypercontractility is a basic property and is independent from other asthma structural cells and airway inflammation, although *in vivo* these may play an important role in modulating the hypercontractile response.

3. Potential Mechanisms Driving ASM Hypercontractility in Asthmatics

3.1. Physiology of Human ASM Contraction. As in all muscle cells, contraction in ASM is initiated by increased cytosolic calcium ions (Ca^{+2}) level, though, unlike most other muscle cells, the source of this Ca^{2+} surge in ASM is mainly from intracellular sarcoplasmic reticulum (SR) stores rather than from the usual extracellular Ca^{2+} influx through voltage-dependent calcium channels during depolarization seen in cardiac, skeletal, and vascular muscle cells.

The sequence of events leading to the contraction of an ASM cell starts with the interaction of a contractile agonist with its G-protein-coupled receptors (Figure 2). This results in the activation of phospholipase C (PLC), which in turn leads to the formation of the inositol triphosphate (IP_3) through the hydrolyzation of phosphatidylinositol bisphosphate (PIP_2). IP_3 then binds to its receptor on SR membrane releasing Ca^{2+} stores which then, through forming a complex with calmodulin, activate myosin light chain kinase (MLCK) which phosphorylates regulatory myosin light chains (rMLC) forming p-MLC [18]. Finally, this leads to the activation of actin and myosin crossbridges resulting in shortening and contraction [19]. After initiation of contraction, cytosolic Ca^{2+} levels return to normal through different mechanisms including pumping out of the cell by the plasma membrane Ca^{2+}-ATPase (PMCA) and the sodium calcium exchanger (NCX), binding to cytosolic proteins, uptake by mitochondria, and also reuptake to the SR through the action of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [20].

Another mechanism for agonist-induced Ca^{2+} released from SR is through ryanodine receptors (RyR). This is mediated by membrane CD38 and nucleotide metabolite cyclic ADP-ribose (cADPR) [21]. The RyR channels are also activated through localized elevation of Ca^{2+} levels (Ca^{2+} induced Ca^{2+} release).

The phosphorylation of rMLC is also regulated by myosin light chain phosphatase (MLCP) which converts p-MLC back to inactive rMLC. MLCP activity is modulated through two agonist-induced mechanisms in a process called calcium sensitization. First, it is controlled by the inhibitory action of diacylglycerol (DAG), another second messenger, which also results from the hydrolyzation of PIP_2. DAG activates Protein Kinase C (PKC) which in turn inhibits MLCP through phosphorylation. Second, MLCP is also negatively controlled by RhoA and its target Rho Kinase, which deactivates MLCP similarly through phosphorylation.

Exploring the possible mechanisms of asthmatic ASM hypercontractility is a difficult task as the evidence is less well established with relatively few human studies. Hypercontraction of asthmatic ASM could be due to abnormalities in one or more of these components or steps of ASM contraction model. The complexity of investigating differences in signalling or contractile proteins is that the abnormality could be in a number of levels. This could be at gene, gene expression (epigenetics), or, more commonly, at protein phosphorylation level.

3.2. Abnormalities of Contractile Proteins. The most characterized potentially abnormal component of the contraction apparatus in asthmatic ASM is MLCK, a key regulator of ASM contraction. Increased MLCK levels have been reported in sensitized animal and human airways [22, 23]. As part

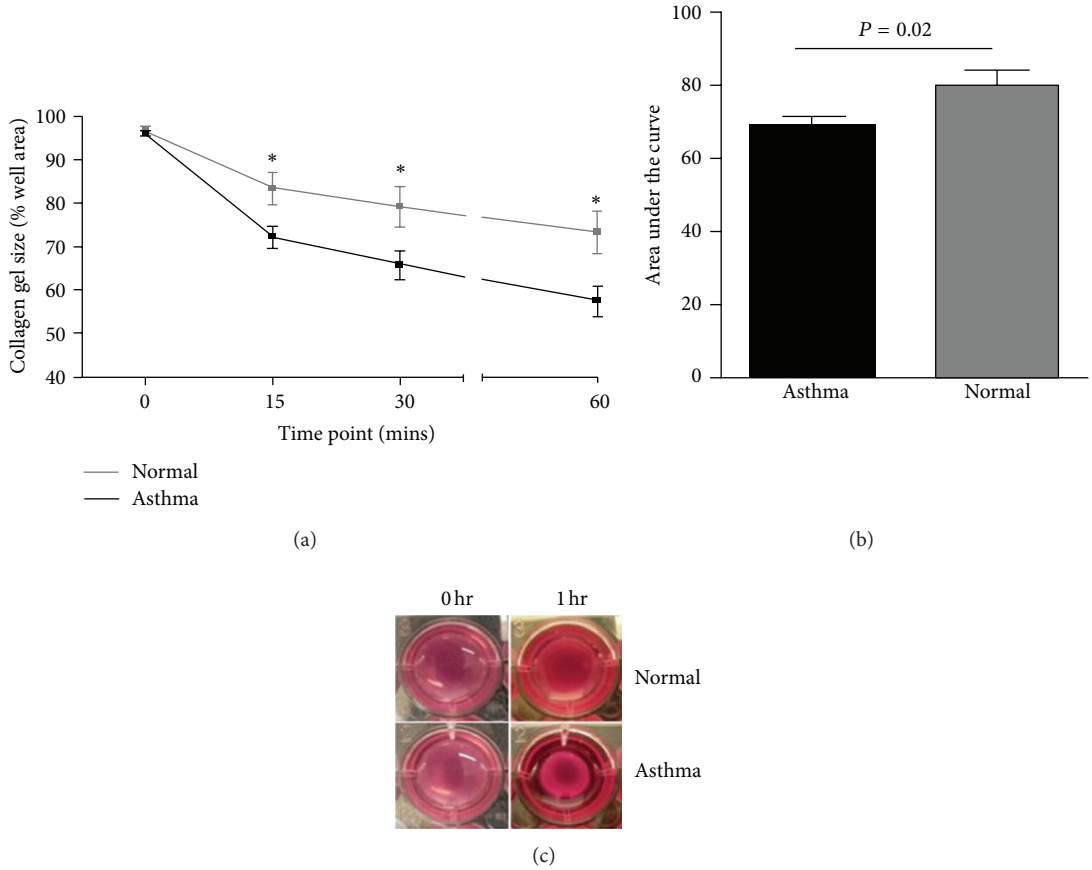

Figure 1: (a) Percentage contraction of collagen gels impregnated with airway smooth muscle from donors with asthma ($n = 19$) versus healthy control donors ($n = 8$) over 1 hour following stimulation with 1 nm bradykinin, (b) area under the curve gel contraction (mean [SEM]), and (c) representative gel photographs taken at 0 hour and 1 hour time points. The comparison was made by unpaired t-test. $^{*}P < 0.05$. [17]. (Reprinted with permission of the American Thoracic Society. Copyright © 2012 American Thoracic Society. Amanda Sutcliffe, Fay Hollins, Edith Gomez, Ruth Saunders, Camille Doe, Marcus Cooke, R. A. John Challiss, and Chris E. Brightling/2012/ Increased Nicotinamide Adenine Dinucleotide Phosphate Oxidase 4 Expression Mediates Intrinsic Airway Smooth Muscle Hypercontractility in Asthma. American Journal of Respiratory and Critical Care Medicine/Vol. 185/pp 267-274. (An official Journal of The American Thoracic Society).

of the same contractility study described earlier, Ma et al. assessed MLCK expression by using RT-PCR to measure mRNA. Measuring MLCK protein was not possible due to the small cell sample (10–20 cells per subject) [15]. MLCK mRNA was significantly increased in asthmatics compared to a group of both allergic and nonallergic nonasthmatic controls.

Benayoun et al. examined contractile protein expression in biopsies in asthmatics with different asthma severity compared to nonasthmatic controls and also compared to patients with COPD [24]. Although α-actin and myosin heavy chain isoforms (SM1, SM2) expression was similar in all groups, MLCK expression was increased in all patients with asthma and COPD compared to controls. Furthermore, MLCK expression was significantly more in patients with severe asthma compared to all other groups. Interestingly, although p-MLC, the active product from the action of MLCK, was detected only in the asthmatic groups, this was not statistically significant.

Not all studies showed increased MLCK expression or content in asthmatics. Matsumoto et al. demonstrated no increase in MLCK content in cultured asthmatic ASM. The authors did admit that this negative result could be due to possible degradation of MLCK during the harvesting stage of ASM cells [16]. Moreover, Woodruff et al. showed no increased gene expression of any of the contractile proteins MLCK, MCH, SM22, or α-actin in a sample of 11 asthmatics compared to 8 controls, although this could be due to the fact that the asthmatics in this study had only mild disease [25].

In vivo, the degree of mast cell infiltration of ASM, a histopathological feature of asthma [26, 27], has been shown to be positively associated with increased α-actin expression [28]. Moreover, *in vitro* coculture of human ASM with human lung mast cell (HLMC), or β-tryptase, a serine protease released by mast cells following activation, resulted in increased α-actin expression and increased ASM contraction. This has been shown to be mediated through autocrine upregulation of transforming growth factor β1 (TGF-β1) in ASM [28]. Histamine release from mast cells in a piece-meal fashion as demonstrated by mast cells within the ASM bundle *in vivo* by electron microscopy [27] and *in vitro*

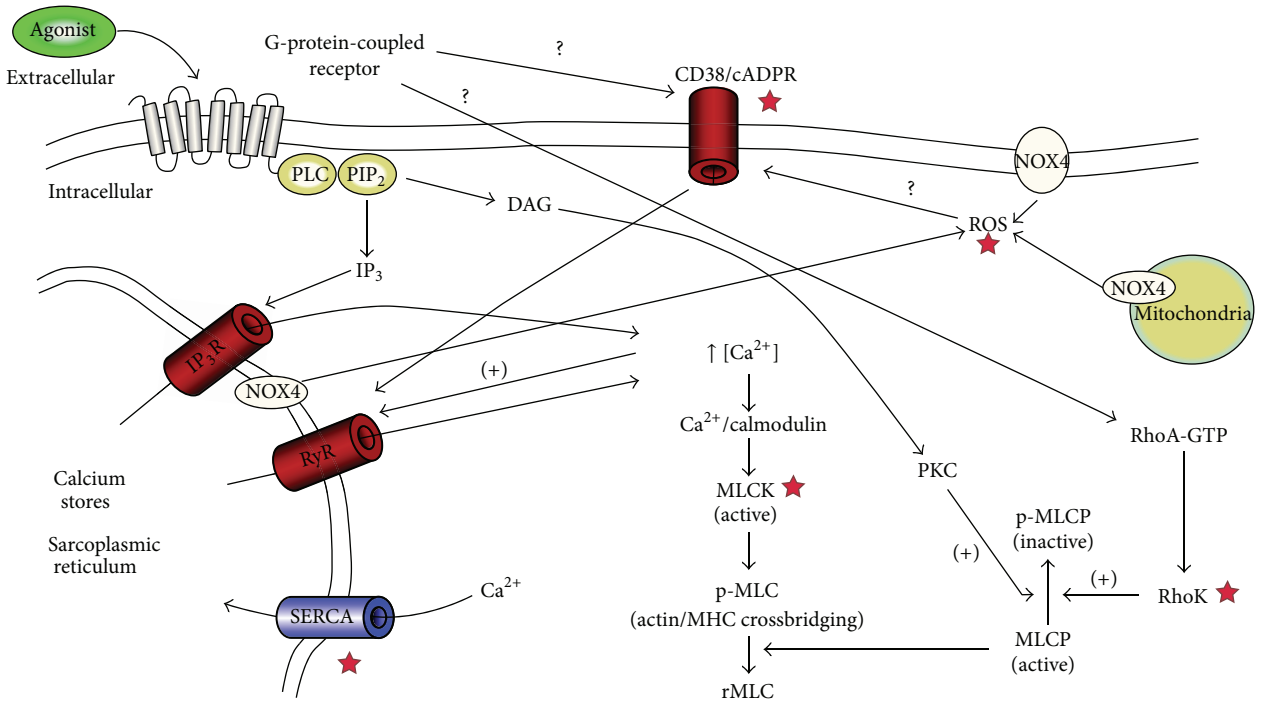

Figure 2: Overview of the signalling pathways involved in airway smooth muscle contraction. The contractile agonist interacts with its specific G-protein-coupled receptor (GPCR) leading to the activation of phospholipase C (PLC) which hydrolyzes phosphatidylinositol bisphosphate (PIP_2) leading to the formation of two-second messengers, inositol triphosphate (IP_3), and diacylglycerol (DAG). IP_3 interacts with its receptor (IP_3R) on the sarcoplasmic reticulum (SR) leading to the release of calcium ions Ca^{2+} which in turn, through forming a complex with calmodulin, activates myosin light chain kinase (MLCK). MLCK phosphorylates regulatory myosin light chain (rMLC) to form p-MLC which leads to myosin and actin crossbridging and contraction. p-MLC is deactivated by the action of myosin light chain phosphatase (MLCP). Both DAG, through its action on protein kinase C (PKC), and RhoA, through its target Rho Kinase (RhoK), have an inhibitory action on MLCP through phosphorylation. Interaction of agonist with GPCR also activates both CD38/cADPR and Rho/RhoK pathways, although the exact mechanism is not fully known. CD38/cADPR activation leads to the release of Ca^{2+} from SR through ryanodine receptors (RyR) channels. Nicotinamide adenine dinucleotide phosphate oxidase type 4 (NOX4) generates reactive oxygen species (ROS) which may affect ASM calcium homeostasis and subsequently ASM contraction through their action on the CD38/cADPR pathway. Red stars ⋆ indicate signalling points with abnormalities suspected of driving hypercontractility in asthmatic ASM.

following fibroblastoid differentiation [29, 30] might exert a direct spasmogen effect upon the ASM in asthma. Mast cells are also an important source of cytokines including the Th2 cytokine IL-13 [31]. Mast cells localized in the ASMbundle express IL-13, particularly in severe disease which can prime ASM to a more hyper-contractile state [31–33]. Abnormalities of α-actin expression in asthmatic ASM have only been described in this context and previous studies examining α-actin expression in asthmatics were mostly negative [24, 34].

3.3. Dysregulation of Calcium Homeostasis. As discussed earlier, calcium plays a central role in ASM contraction. There is an increasing pool of evidence showing abnormal calcium homeostasis in asthmatic ASM, thus, suggesting that abnormal calcium handling, signaling, or storage as possible underlying mechanisms for asthmatic ASM hypercontractility is a plausible argument.

In general, factors leading to increased cytosolic Ca^{2+} levels result in increased ASM contraction. Mahn et al. examined SERCA expression in ASM from asthmatics and healthy volunteers [35]. The expression of SERCA2 isoform mRNA, the main isoform expressed in human ASM, was reduced in patients with moderately severe asthma. This was found in *in vivo* samples and also on ASM cultures. Reduction of SERCA2 expression in healthy control ASM culture by siRNA resulted in phenotypic shifting to an asthmatic ASM type with increased motility, secretion, and slow Ca^{2+} recovery. Of interest, IP_3R mRNA expression was not increased in the asthmatics group.

Another signalling pathway that has the potential of altering calcium homeostasis and increasing contractility is the CD38/cADPR/RyR pathway. CD38 deficiency in animals has been shown to inhibit airway hyperresponsiveness (AHR) [36]. In human ASM, TNF-α, IL-1β, IL-13, and IFN-γ were all found to increase CD38 expression, cADPR activity, and Ca^{2+} response to various natural contractile agonists [37, 38]. Moreover, in another study, highlighting the possibility that CD38 abnormalities could be a fundamental characteristic in asthma, TNF-α was shown to significantly increase CD38 expression in asthmatic ASM than in controls [39].

Altered calcium homeostasis in asthmatic ASM is also due to altered extracellular calcium influx through non-voltage-dependent channels [40]. Although this has been directly implicated in altered mitochondrial biogenesis and increased ASM proliferation in asthmatics, its relevance to hypercontractility remains to be investigated.

3.4. Abnormal Calcium Sensitization. Upregulation of the calcium independent RhoA/Rho Kinase signalling pathway leading to inhibition of MLCP would result in increased levels of p-MLC and subsequently increased ASM contraction force at the same Ca^{2+} concentration. Abnormalities of this signalling pathway have been described in animal models of various smooth muscle disorders including hypertension, coronary artery spasm, and preterm labour [41]. This has also been described in animal models of allergic bronchial asthma [42]. Increased levels of RhoA protein and RhoA mRNA were found in airway hyperresponsive rat models although this is probably medicated through inflammatory cytokines [41–43].

We emphasize that most of the abnormalities of calcium homeostasis and calcium sensitization explored in this paper were only described in single studies which have not been replicated; thus their importance remains to be fully determined.

3.5. Increased Oxidative Stress Burden. Although reactive oxygen species (ROS) play an important physiological role in different cellular functions, excessive production results in the tissue damage seen in a range of chronic and acute diseases. Oxidative stress burden is increased in bronchial asthma with recent evidence identifying an increase in the generation of ROS in asthmatic ASM *in vivo* and in primary ASM cultures [17]. This was inversely correlated to the degree of airflow obstruction and AHR. More importantly, nicotinamide adenine dinucleotide phosphate oxidase type 4 (NOX4) expression, an important source of ROS, was increased in asthmatics ASM. Moreover, increased asthmatics ASM contractility seen in gel contraction essay was abolished by adding NOX4 inhibiters or NOX4 small interfering RNA.

3.6. SMAD3 and ORMDL3. Genome-wide association studies (GWASs) have identified several associations between multiple single-nucleotide polymorphisms (SNPs) on a number of locations and asthma. One such SNP is on the *SMAD3 gene*. *SMAD3 gene,* located on chromosome 15, encodes for a similarly named protein, SMAD3 protein. This protein is a signal transduction and transcription modulator and is activated by TGF-β which has a complex role in cell growth and proliferation and is involved in airway inflammation and remodelling in asthma. As mentioned earlier, upregulation of TGF-β1 observed on coculture of ASM and HLMC was associated with increased α-actin expression and increased contractility of ASM [28].

Another association identified by GWASs was on the *ORMDL3 gene* [44, 45]. This gene encodes for the SR membrane protein ORMDL3 which is thought to have an important role in calcium homeostasis possibly through its action on SERCA. Overexpression of ORMDL3 was found to be associated with reduced SERCA activity as evidenced by higher basal cytosolic Ca^{2+} levels, lower SR Ca^{2+} levels, and slower Ca^{2+} reuptake into the SR [46]. Thus, based on the above evidence, polymorphism of *SMAD3* or *ORMDL3* could be implicated in the hypercontractility seen in asthmatic ASM through their effect on α-actin expression and calcium homeostasis, respectively.

4. Clinical Relevance of Asthmatic ASM Hypercontraction

The contraction of ASM in asthma causes airway obstruction [47]. Although we know that AHR is predominantly a function of ASM, how much of it is driven by ASM hypercontractility is a difficult and controversial question to answer. We do recognize that some of ASM hypercontractility is contributed to by airway inflammation and inflammatory cytokines, but there is also good evidence to suggest that there is more to AHR than inflammation. Although corticosteroids reduce AHR, studies using agents that target specific parts of inflammation, in the form of antibodies against IL-5 and IgE, significantly improved inflammation but did not affect AHR [48–50].

Therapies that reduce ASM contraction have been shown to improve asthma symptoms, lung function, and maybe even AHR. The roles of β2-agonists and anticholinergics in the treatment of asthma are well established. Another evidence of the significance of ASM is from bronchial thermoplasty (BT). BT, where radiofrequency energy is used on airways to reduce ASM mass, was shown to improve asthma control, quality of life, and, in one study, AHR [51]. Further studies are required to examine whether the efficacy of BT in asthmatics is related to changes in ASM mass and how this relates to changes in airway structure, physiology, and clinical expression of disease.

5. ASM Hypercontractility and the Future of Asthma Therapy

The desperate need for new asthma treatments is universally acknowledged. Asthma incidence is increasing with more than half of the patients failing to achieve adequate control. Furthermore, 5–10% of patients have persistent symptoms despite maximal treatment with conventional anti-inflammatory and bronchodilator therapy [1, 52].

Detailed discussion of the future of asthma treatment is beyond the scope of this paper. Targeted drugs that would act on specific aspects of inflammation and contractility are the way forward. Over the last few years, a huge research effort has been on trying to identify asthma phenotypes based on inflammation, but much less was dedicated to addressing contractility. Several chemicals have been found to reduce ASM gel contraction *in vitro* including inhibitors of phospholipase C, myosin light chain kinase, Rho kinase, and NOX4 and thus this has identified these enzymes as potential targets for future novel asthma treatments [16, 17].

6. Conclusion

In conclusion, we believe, based on the evidence reviewed, that ASM in asthmatics is hypercontractile. Airway inflammation contributes to and augments ASM hypercontractility but is neither sufficient nor necessary. Indeed evidence presented here suggests that ASM hypercontractility is an intrinsic abnormality in asthma that persists in primary culture in the absence of the asthmatic environment. Improved understanding of the mechanisms driving this hypercontractility will pave the way for future treatments that will address contractility, achieve better relief of airway obstruction, and impact on asthma control and exacerbations.

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Review Article

Accumulating Evidence for Increased Velocity of Airway Smooth Muscle Shortening in Asthmatic Airway Hyperresponsiveness

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It remains unclear whether airway smooth muscle (ASM) mechanics is altered in asthma. While efforts have originally focussed on contractile force, some evidence points to an increased velocity of shortening. A greater rate of airway renarrowing after a deep inspiration has been reported in asthmatics compared to controls, which could result from a shortening velocity increase. In addition, we have recently shown in rats that increased shortening velocity correlates with increased muscle shortening, without increasing muscle force. Nonetheless, establishing whether or not asthmatic ASM shortens faster than that of normal subjects remains problematic. Endobronchial biopsies provide excellent tissue samples because the patients are well characterized, but the size of the samples allows only cell level experiments. Whole human lungs from transplant programs suffer primarily from poor patient characterization, leading to high variability. ASM from several animal models of asthma has shown increased shortening velocity, but it is unclear whether this is representative of human asthma. Several candidates have been suggested as responsible for increased shortening velocity in asthma, such as alterations in contractile protein expression or changes in the contractile apparatus structure. There is no doubt that more remains to be learned about the role of shortening velocity in asthma.

1. Introduction

It has long been known that deep inspirations (DI) in healthy subjects have both bronchoprotective and bronchodilating effects. In asthmatics however these effects are reduced, and in some cases DI exacerbate breathing difficulties. A recent study by Jackson et al. [1] measured the rate at which airway resistance is regained after a DI in methacholine challenged individuals. In asthmatics this rate was significantly higher. Furthermore, while both asthmatics and controls had reduced airway resistance directly after DI, a few minutes later airway resistance in asthma was often increased relative to pre-DI levels, while healthy subjects' airway resistance remained lower. This difference in the rate at which airway resistance is regained is hard to explain with traditional ideas of how airway smooth muscle (ASM) contributes to airway hyperresponsiveness (AHR), that is, increased ASM mass and/or increased ASM force. While non-smooth muscle factors such as parenchymal interdependence may also explain these effects, in the following we argue that an increased ASM velocity of shortening may be responsible for the differential responses between healthy and asthmatic subjects. The rate of shortening of ASM is dependent on the crossbridge cycling rate, the organization of contractile elements within the muscle cells and the internal loading of the ASM cell. The crossbridge cycling rate is high in the early phase of contraction but decreases as the contraction progresses, allowing for the maintenance of force at low energy cost (i.e., latch) [2]. Solway and Fredberg have suggested that smooth muscle with a higher shortening velocity may not benefit as much from the ASM stretches caused by tidal breathing and deep inspirations, as the increased shortening velocity may lead to a faster return to a prestretch length [3]. Furthermore, if an increased shortening velocity is caused by an increase in contractile elements placed in series, the absolute strain and consequently the force per contractile element will be reduced as the strain is distributed over

more elements. Consequently, crossbridge cycling will be less affected by strain, resulting in a faster regaining of airway resistance after a DI.

Our lab set to explore the possibility that an increased shortening velocity in asthma could lead to increased airway constriction under physiological circumstances [4]. In rat trachealis muscle exposed to sinusoidal force oscillations resembling breathing and an occasional DI, we showed that increased shortening velocity correlated with increased total shortening of the muscle and faster recovery after DI. Furthermore, we found that an increase in shortening velocity is as effective, if not more effective, as an equal increase in contractile force. Accordingly, increased airway constriction in asthma does not require increased force generation: an increased shortening velocity would suffice.

Establishing whether shortening velocity changes occur in, and contribute to, asthma is a major challenge. Not only is it difficult to get human ASM, but the interpersonal variability in mechanical response is so large that any but the largest changes will not be noticed [5–7]. Furthermore, it is known that ASM mechanical properties are different for different regions in the lungs [8]. To complicate things further, the measurement of shortening velocity is prone to large variability because of the different procedures used by different labs, the effects of differing methods of force control, the changing velocity with contraction duration and agonist type, and the accuracy of the measurement equipment itself. In this paper we will discuss the varying approaches that have been taken by us and other researchers to assess if and how shortening velocity in asthma is increased and the associated challenges and reservations with each approach.

2. Is Shortening Velocity Increased?

Ideally, to confirm an increase in shortening velocity in asthma, ASM tissue from asthmatics and controls are used to assess the shortening velocity directly. However, besides the above-mentioned reservations, shortening velocity varies with age [9] and location in the lung [8] and is likely to be affected by genetic differences and environmental factors. As the procedure for procuring lung biopsies is not harmful to the subject, biopsies can be obtained under controlled conditions with the benefit of having access to detailed medical history and respiratory function parameters and the possibility of collecting data from groups of subjects with similar disease history and background. However, biopsies cannot provide muscle strips, only cells. Bronchial smooth muscle cells from asthmatics have shown an increased rate of shortening and total shortening when exposed to contractile agonists compared to controls [10]. However, the process of obtaining the biopsies and dissociating the cells is likely to have a direct effect on the mechanical response. In addition, the mechanical response of the ASM cell in isolation may not directly correlate with ASM tissues or airways *in situ*. In fact, the total extent of shortening of the unloaded cells is many times greater than is likely to occur *in vivo* and most of this rapid shortening may occur before the cell is fully activated [11]. Consequently, the difference in shortening velocity may be a difference in the rate of activation instead.

Entire bronchial rings or tissue strips can be obtained from excess tissue from lung resections and pneumonectomies, with a downside of lack of control over subject health status and age. Many studies have been performed on human bronchial rings, but only one has looked at shortening velocity directly [7]. In this study, bronchial rings from nonasthmatic subjects were sensitized overnight with human serum from atopic individuals. While the maximal force remained constant, the shortening velocity and the total amount of shortening increased in sensitized bronchi. This study does not directly prove that shortening velocity is increased in asthma; however, it does suggest a mechanism by which shortening velocity could be increased without changing the force generating capacity of ASM.

Another approach that has been used to study the pharmacology of bronchoconstriction, and which also yields information about the shortening velocity, is the airway explant [12–15]. In this technique, lungs filled with agarose are sliced transversely and placed under a microscope. The rate of narrowing in response to methacholine has been measured in the Fisher and Lewis rat model of AHR and asthma; a greater rate of shortening was reported in the hyperresponsive Fisher rat [16]. While the lung slice technique has been used to study the dynamics of bronchoconstriction in human airways [17], asthmatic airways have not yet been investigated. One disadvantage of this method of studying ASM mechanics is that it limits the axial continuity of the ASM bundles, resulting instead in patches of ASM cells. The orientation of the muscle bundles may have a profound effect on resistance to airway constriction, and as such the ASM shortening velocity.

Nowadays, entire donor lungs can be used for research when no suitable transplant recipient can be found (Figure 1). The main benefit of transplant lungs is that repeated investigation of tissues from the same sections in the lung can be performed, reducing at least some of the variability between subjects found in tissues from (partial) lobectomies. Furthermore, as most ASM studies in animals are done on trachealis muscle, the availability of the trachea allows for a more direct comparison with these results. A study by Chin et al. [5] on human trachealis muscle showed a nonsignificant trend of increased shortening velocity in asthmatics. the lack of significant differences seems to be related to large variability in the data, combined with a substantial age difference between the subject groups. As no follow-up studies on respiratory function are possible, it is difficult to assess the quality of the lungs and their position in the healthy-to-severe asthma spectrum. Furthermore, as research centres are generally not in the direct proximity to the tissue source, a considerable delay exists between the harvesting of the lung and the experiments. Studies on isolated airway segments have shown little change in pharmacological response of bronchi stored for more than 2 days [6], but little is known about the effect on ASM of prolonged storage with surrounding tissues (i.e., parenchyma, blood traces, etc.).

Many studies on animal models of AHR have shown an increase in shortening velocity [18, 19]. However, as the real

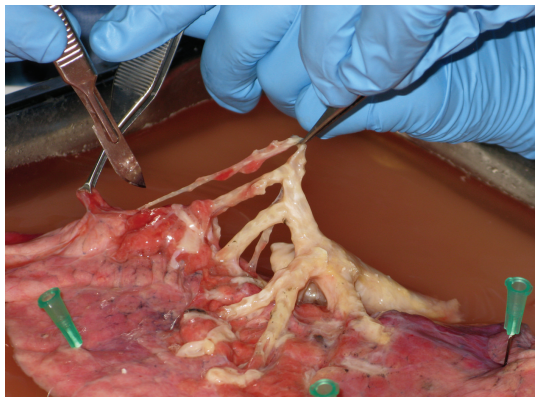

Figure 1: Single lobe dissection of human lung. ASM mechanics from trachea to small bronchi (~1 mm diameter) from both healthy and asthmatic subjects may lead to more conclusive evidence of shortening velocity changes in asthma.

cause of asthma is as yet unknown, no animal model can be said to mimic asthma, only asthma symptoms. As such, it is irrelevant whether these animal models show increased shortening velocity, what matters is what causes it and does evidence for similar pathways exist in humans.

3. How Is Shortening Velocity Changed?

If increased shortening velocity does play a role in the pathophysiology of asthma, what is responsible for this increase? Molecular level changes could result in faster cross-bridge cycling rates, either through changes in the contractile elements themselves, or through changes in the level of activation. The smooth muscle myosin heavy chain isoform SM-B has been shown to result in a doubling of velocity in motility assays compared to SM-A [20]. The ratio of SM-B/SM-A mRNA is increased in asthmatics [21] but this has yet to be followed by measurements of the protein levels. Furthermore SM-B deficient mice have a decreased rate of airway constriction after methacholine challenge [22]. The crossbridge cycling rate could also be changed independently from differences in myosin isoforms. The gradual change in shortening velocity during a sustained contraction is a strong indicator of variable cycling rates, and most evidence is pointing towards a pivotal role for the myosin light chain phosphorylation level in determining crossbridge cycling rates [2, 23]. Indeed, myosin light chain kinase (MLCK), which phosphorylates the myosin light chain, is increased in asthma at the mRNA level [10, 21] and a variant of the MLCK gene has been associated with severe asthma in African Americans [24]. Furthermore, human bronchi sensitized with serum from allergic asthmatic individuals show increased MLCK levels [25]. An alternative, but not yet tested, theory involves the lengths of the contractile elements within the ASM cells, and its thin filaments in particular. Assuming that the unloaded shortening velocity of an individual contractile element is independent of its length, the total rate of shortening of a cell is determined by the effective number of these contractile elements in series. As ASM is known to allow rapid remodelling, changes in the series to parallel organization of contractile elements are likely to occur, and this is required to explain the constant force at a wide range of lengths in most mathematical models of length adaptation [26–28]. Consequently, a change in the average thin filament length could result in a change in shortening velocity of the muscle [27, 28].

Evidence is mounting that the cause for the changes in ASM shortening velocity may have its roots in chronic airway inflammation. Chronic airway inflammation and remodeling underlie the clinical manifestations of asthma. Cultured ASM cells exhibit enhanced contractility and contractile protein expression in response to a number of important cues altered in asthma, including inflammatory mediators [29]. Studies have suggested that airway inflammation is causally related to AHR and these changes could be a result of $CD4^+$ T cell activation, which is an important source of inflammatory mediators. An increase in the number of $CD4^+$ T cells with a phenotype associated with T-cell activation is found both in bronchoalveolar lavage of asthmatic patients [30] and in the ASM layers of animals with experimental asthma [31]. Lazaar and coworkers [32] originally demonstrated that activated T cells can adhere in vitro to resting ASM cells from nonasthmatic patients. The adhesion was enhanced when ASM cells were primed with proinflammatory cytokines such as tumor necrosis factor-α (TNF-α). These findings independently confirmed that $CD4^+$T cells can interact with ASM not only in vitro [33] but also in vivo [31]. Recently, it has been shown that IL-17A, produced by $CD4^+$ T cells, enhanced contractile force generation of human ASM through an IL-17 receptor A [34]. This pathway involves activation of NF-κB (a protein complex that controls the transcription of DNA) and induction of RhoA and ROCK2 expression. ROCK 2 regulates myosin light chain 20 (LC_{20}) phosphorylation through inhibition of myosin light chain phosphatase thus promoting the phosphorylated level of LC_{20}. These data correlate with studies of Fan and others [19] who found the increasing of MLCK activity and phosphorylation of LC_{20} due to incubation of muscle strips with different inflammatory mediators (including Th2 cytokines) might result in the observed increase of shortening velocity.

4. Conclusion

While an increased shortening velocity might not be the only change occurring in asthmatic ASM, it may certainly play a central role in the pathophysiology of asthma. More research is needed to conclusively determine whether, and by how much, shortening velocity is increased in asthma, but the various approaches taken so far provide a very strong indication for an increase as well as likely causes for the increase. Future important findings will probably come from running experiments on tracheal, main bronchial, and intrapulmonary bronchial tissues from whole lungs from transplant programs to assess whether shortening velocity in asthma is changed (Figure 1). Advances in cantilever microfabrication [35], for example, will allow the assessment of loaded cell mechanics by adhering ASM cells directly to

a length and a force transducer. This more direct assessment, combined with control experiments on whole ASM tissues, will show whether these cell measurements are indicative of whole-tissue behaviour. This will greatly extend the power of biopsy samples. Furthermore, human lung explants [12–15] could be used for a direct comparison of asthmatic and control airway shortening velocity. All this together will shed light on whether or not the velocity of shortening of ASM is altered in asthma. At this point, some doubt remains especially after the lack of shortening velocity change found recently by Chin et al. [5]. Perhaps future ASM tissue studies can reduce the variability and result in a clearer conclusion on whether shortening velocity is really increased in asthma.

Acknowledgments

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Review Article

Altered CD38/Cyclic ADP-Ribose Signaling Contributes to the Asthmatic Phenotype

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CD38 is a transmembrane glycoprotein expressed in airway smooth muscle cells. The enzymatic activity of CD38 generates cyclic ADP-ribose from β-NAD. Cyclic ADP-ribose mobilizes intracellular calcium during activation of airway smooth muscle cells by G-protein-coupled receptors through activation of ryanodine receptor channels in the sarcoplasmic reticulum. Inflammatory cytokines that are implicated in asthma upregulate CD38 expression and increase the calcium responses to contractile agonists in airway smooth muscle cells. The augmented intracellular calcium responses following cytokine exposure of airway smooth muscle cells are inhibited by an antagonist of cyclic ADP-ribose. Airway smooth muscle cells from CD38 knockout mice exhibit attenuated intracellular calcium responses to agonists, and these mice have reduced airway response to inhaled methacholine. CD38 also contributes to airway hyperresponsiveness as shown in mouse models of allergen or cytokine-induced inflammatory airway disease. In airway smooth muscle cells obtained from asthmatics, the cytokine-induced CD38 expression is significantly enhanced compared to expression in cells from nonasthmatics. This differential induction of CD38 expression in asthmatic airway smooth muscle cells stems from increased activation of MAP kinases and transcription through NF-κB, and altered post-transcriptional regulation through microRNAs. We propose that increased capacity for CD38 signaling in airway smooth muscle in asthma contributes to airway hyperresponsiveness.

1. Introduction

Contractility of airway smooth muscle (ASM) depends on the dynamic regulation of intracellular calcium concentration [1]. Contractile agonists act on G-protein-coupled receptors to cause oscillatory changes in $[Ca^{2+}]_i$, mediated by influx of calcium from the extracellular space and release of calcium from the intracellular stores [2–5]. The major $[Ca^{2+}]_i$ store in ASM cells is the sarcoplasmic reticulum (SR). Release of calcium from the SR is brought forth principally by two signaling molecules, inositol 1,4,5-trisphosphate (IP_3) and cyclic ADP-ribose (cADPR). Cyclic ADP-ribose is derived from β-NAD through the enzymatic activity of ADP-ribosyl cyclase which is a constituent of the cell-surface protein CD38 [6] (reviewed in [2]). Calcium influx into the cell can occur through voltage- and receptor-operated calcium channels in the plasma membrane [7, 8]. It can also occur by influx that is triggered by depletion of SR calcium stores through a mechanism known as store-operated calcium entry [4]. Transient receptor potential proteins are thought to mediate influx of calcium through receptor- and store-operated channels [9].

In ASM cells, contractile agonists cause a biphasic elevation of $[Ca^{2+}]_i$ that is characterized by an initial rapid rise, followed by a decline to a plateau concentration above the basal level. The initial rapid phase of the biphasic elevation of $[Ca^{2+}]_i$ has been attributed to SR calcium release, while the sustained phase of elevation is to influx from extracellular space. However, with the use of improved temporal and spatial resolution features of real-time confocal microscopy, our understanding of agonist elicited $[Ca^{2+}]_i$ elevation has significantly changed. These studies have demonstrated that

the biphasic elevation of the $[Ca^{2+}]_i$ consists of propagating regenerative calcium oscillations of similar amplitude within a given region of the ASM cell. The frequency and the propagation velocity of these calcium oscillations increase with increasing concentration of the agonist. The biphasic nature of the $[Ca^{2+}]_i$ response is in fact the spatiotemporal integration of oscillations in calcium across the entire cell. While the initiation of the calcium oscillations depends on SR calcium release, their sustenance requires repletion of the SR stores through influx from the extracellular space. Furthermore, activation of the IP_3 receptors in the SR is crucial for the initiation of calcium oscillations by agonists, while maintained calcium oscillations require calcium release through the ryanodine receptor channels in the SR. Calcium release through the ryanodine receptor channels in the SR in ASM cells is mediated by cADPR. Recent studies have also demonstrated that inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ and the Th2 cytokine IL-13 regulate the expression and function of the pathways that govern $[Ca^{2+}]_i$ responses to agonists, thereby contributing to ASM hyperresponsiveness [10, 11]. One of the pathways in $[Ca^{2+}]_i$ regulation in ASM cells involves CD38, and the expression of CD38 in ASM cells derived from asthmatics is upregulated by TNF-α to a significantly greater extent than in cells from nonasthmatics [12]. The regulation of CD38 expression and the role of different transcription factors, signaling intermediates and microRNAs in this regulation, have been the focus of investigations in our laboratory.

CD38 is a 45-kDa type II glycoprotein expressed in a variety of cells from a diverse array of organisms. It belongs to a family of nucleotide metabolizing enzymes capable of generating cyclic adenosine diphosphoribose (cADPR) and ADPR from β-NAD or NAADP from NADP [13]. cADPR, ADPR, and NAADP have been shown to be involved in intracellular calcium regulation in both immune cells and excitable cells such as smooth muscle cells. Earlier studies from our laboratory showed evidence for intracellular calcium release by cADPR during activation of $G_{\alpha q}$ and $G_{\alpha i}$-type G-protein-coupled receptors in airway smooth muscle cells [14]. Studies in other types of cells indicate that this calcium release involves the dissociation of FKBP-12.6 and activation of ryanodine receptors in the sarcoplasmic reticulum [15, 16]. Evidence from FKBP-12.6 knockout mice revealed that cADPR may be an endogenous ligand for this protein and binding of cADPR is an essential step in the activation of ryanodine receptor calcium release channels [17]. In airway smooth muscle cells as well as cardiac myocytes, the calcium-induced calcium release mechanism may be mediated through activation of ryanodine receptor channels by cADPR. The evidence that cADPR is involved in calcium release in airway smooth muscle stems from the following observations: direct addition of cADPR to the cytosolic compartment of airway smooth muscle cells releases calcium from ryanodine receptor channels [18]; cADPR antagonists inhibit intracellular calcium release brought forth by contractile agonists [14]; increasing CD38 expression by inflammatory cytokines (i.e., TNF-α, IL-1β, IL-13) and thereby augmenting CD38/cADPR signaling cause significant enhancement of calcium release by agonists that is sensitive to inhibition by cADPR antagonists [10]. These results provide evidence for CD38/cADPR signaling in the regulation of intracellular calcium and its potential for enhanced contribution to such regulation during inflammation in airway smooth muscle.

Investigations from other laboratories show that in chemokine-stimulated neutrophils and dendritic cells, ADPR may activate plasma membrane-associated TRPM2 calcium channels and thereby regulate neutrophil and dendritic cell chemotaxis [19]. Evidence from Lund's laboratory has revealed that chemotaxis and bacterial clearance are significantly compromised in neutrophils obtained from CD38 deficient mice [20]. Furthermore, evidence also demonstrates that in CD38 deficient mice there is significant attenuation of T-cell dependent humoral immune responses following immunization with antigens [21]. This defect in humoral immune response appears to result from lack of migration of dendritic cells from inflammatory sites to regional lymph nodes and insufficient dendritic cell priming of CD4 T cells at these sites [20]. These results provide evidence for a role of CD38 in both innate and adaptive immune responses of the host.

2. CD38/cADPR Signaling and Airway Smooth Muscle Function

In an attempt to elucidate the contribution of this signaling pathway to airway function, we developed two different models systems to evaluate intracellular calcium responses to spasmogens: ASM cells obtained from CD38 knockout and wild-type mice; human ASM cells expressing a smooth muscle phenotype and maintained in short-term cultures. The intracellular calcium responses to acetylcholine and endothelin-1 of cells obtained from CD38KO mice were significantly lower than responses in cells from WT mice [22]. The calcium responses in the myocytes from CD38KO mice were also insensitive to modulation by the cADPR antagonist, indicating that the defect in calcium signaling can be attributed to lack of cADPR generation [22]. This defective calcium signaling in airway smooth muscle cells is reflected in significant attenuation of methacholine-induced airway resistance and dynamic compliance measured in intact CD38KO mice, suggesting an airway phenotype of these mice [22].

In HASM cells in culture, the intracellular calcium responses to multiple spasmogens were found to be significantly augmented upon treatment with inflammatory cytokines that are implicated in asthma [10]. The augmented calcium responses were attributable to increased CD38/cADPR signaling since they were reduced by a cADPR antagonist as well as by antisense downregulation of CD38 expression [10, 23]. The fact that inflammatory cytokines such as TNF-α and IL-13, a Th2 cytokine, are capable of increasing the capacity for CD38/cADPR signaling in human ASM cells indicates its potential role in human asthma.

Based on the results reported above in the CD38KO mice and in HASM cells following exposure to inflammatory cytokines, we hypothesized that CD38/cADPR signaling will

be enhanced during airway inflammation, thus contributing to airway hyperresponsiveness, a hallmark feature of asthma. In order to address this hypothesis specifically, we used two different model systems: (i) mouse models of inflammatory airway disease to assess the contribution of CD38 to airway inflammation and AHR; (ii) CD38 expression, function, and its regulation in ASM cells obtained from asthmatic and nonasthmatic donors.

3. Contribution of CD38 to the Asthmatic Phenotype

CD38 deficient mice were generated by Cockayne et al. to study the role of this molecule in host immune responses against pathogens [21]. $CD38^{-/-}$ mice showed an immunological phenotype characterized by attenuated chemotaxis and antigen presentation by dendritic cells [20]. In our laboratory, we evaluated the respiratory phenotype of $CD38^{-/-}$ mouse following induction of airway inflammation by murine recombinant IL-13 or TNF-α. The inflammatory cytokine TNF-α and the Th2 cytokine IL-13 play important roles in the development of allergic asthma. In light of the findings by Partida-Sánchez et al. that CD38-null mice show suboptimal inflammatory response [20], we chose the model of cytokine-induced airway inflammation to circumvent the possibility of reduced inflammatory response to allergen challenge. Following brief and repeated exposure to IL-13 or TNF-α, comparable airway inflammation was induced in wild type and $CD38^{-/-}$ mice [24, 25]. However, the airway resistance in response to the contractile agonist methacholine was significantly attenuated in the $CD38^{-/-}$ mice compared to the wild type mice [24, 25]. In light of our findings that CD38 deficient mouse airway myocytes show reduced Ca^{2+} responses, we hypothesized that the differential methacholine responsiveness of $CD38^{-/-}$ mice can be attributed to the altered ASM contractility. Tracheal rings isolated from the WT and $CD38^{-/-}$ were used for measurement of isometric contractile responses following exposure to IL-13 or TNF-α *in vitro*. CD38-deficient tracheal rings generated significantly reduced isometric force in response to agonist compared to the tracheal rings from WT mice [24, 25]. These observations support our hypothesis that CD38 contributes to the development of airway hyperresponsiveness (AHR) through its pivotal role in ASM Ca^{2+} dynamics and contractility.

Based on the findings reported by Lund and her coworkers, the immunological functions of CD38 in the development of AHR cannot be understated. Studies by Lund showed that CD38 expression in immune cells is critical for the inflammatory and immunological steps that culminate in allergy and AHR (reviewed in [26]). The critical events in host immune response to allergens, such as antigen presentation by dendritic cells, were negatively impacted in CD38-deficient mice [20]. Studies using ovalbumin-induced allergic inflammation in mouse revealed that along with attenuated methacholine responsiveness, CD38-deficient mice also developed reduced airway inflammation, signified by substantially reduced eosinophilia, Th2 cytokines, and allergen-specific immunoglobulins E (IgE) and G1 (IgG1) levels compared to the WT mice [27]. These findings support a pivotal role for CD38 in the pathogenesis of asthma through its dual functions in the immune response to allergen and ASM contractility. The involvement of CD38/cADPR signaling pathway in the pathogenesis of asthma at multiple levels of the process is supported by these observations.

4. Asthmatic Phenotype of Airway Smooth Muscle

The intracellular calcium responses to contractile agonists, isometric contractile responses of tracheal rings, and responsiveness of airways to inhaled methacholine are significantly attenuated in airway smooth muscle obtained from $CD38^{-/-}$ mice. In HASM cells exposed to inflammatory cytokines, the CD38/cADPR signaling pathway contributes to enhanced calcium signaling in response to activation of G-protein-coupled receptors [10]. The airway hyperresponsiveness following cytokine challenge or allergen sensitization and challenge is significantly compromised in $CD38^{-/-}$ mice [10]. These observations led us to speculate that ASM cells derived from asthmatic donors will have increased basal and cytokine-induced expression of CD38. To address this hypothesis, we obtained ASM cells from nonasthmatic and asthmatic donors. The cells were maintained in culture for up to 5 passages. Cells were exposed to TNF-α or vehicle following growth arrest and CD38 expression, and their enzymatic activities were measured. There was very little, if any, basal expression of CD38 in cells from either asthmatic or nonasthmatic donors [12]. However, exposure to a range of TNF-α concentrations caused a significantly greater induction of CD38 expression in ASM cells from asthmatics than in cells from nonasthmatics [12]. This differential induction of CD38 expression occurred as early as 6 hrs following exposure to TNF-α and maintained for over 24 hrs and was unrelated to level of TNFR1 expression in the cells (Figure 1) [12]. This pattern of differential induction of CD38 expression was seen in cells obtained from donors with a history of clinical asthma as well as from fatal asthmatics. This asthmatic phenotype in terms of differential CD38 induction was maintained for up to 5 passages in culture.

We examined some potential mechanisms involved in the differential induction of CD38 expression and for the asthmatic phenotype of ASM cells. Our previous studies showed that members of the MAP kinases and the transcription factors NF-κB and AP-1 were required for TNF-α induction of CD38 expression in HASM cells [29]. Therefore, we hypothesized that the increased CD38 expression will be reflected by greater activation of MAP kinases and the transcription factors NF-κB and AP-1. It is worth noting that the *CD38* promoter has response elements for these transcription factors and mutagenesis of either of these transcription factor binding sites would result in lack of promoter activation by TNF-α [30]. In HASM cells derived from asthmatic donors, we found consistently elevated p38

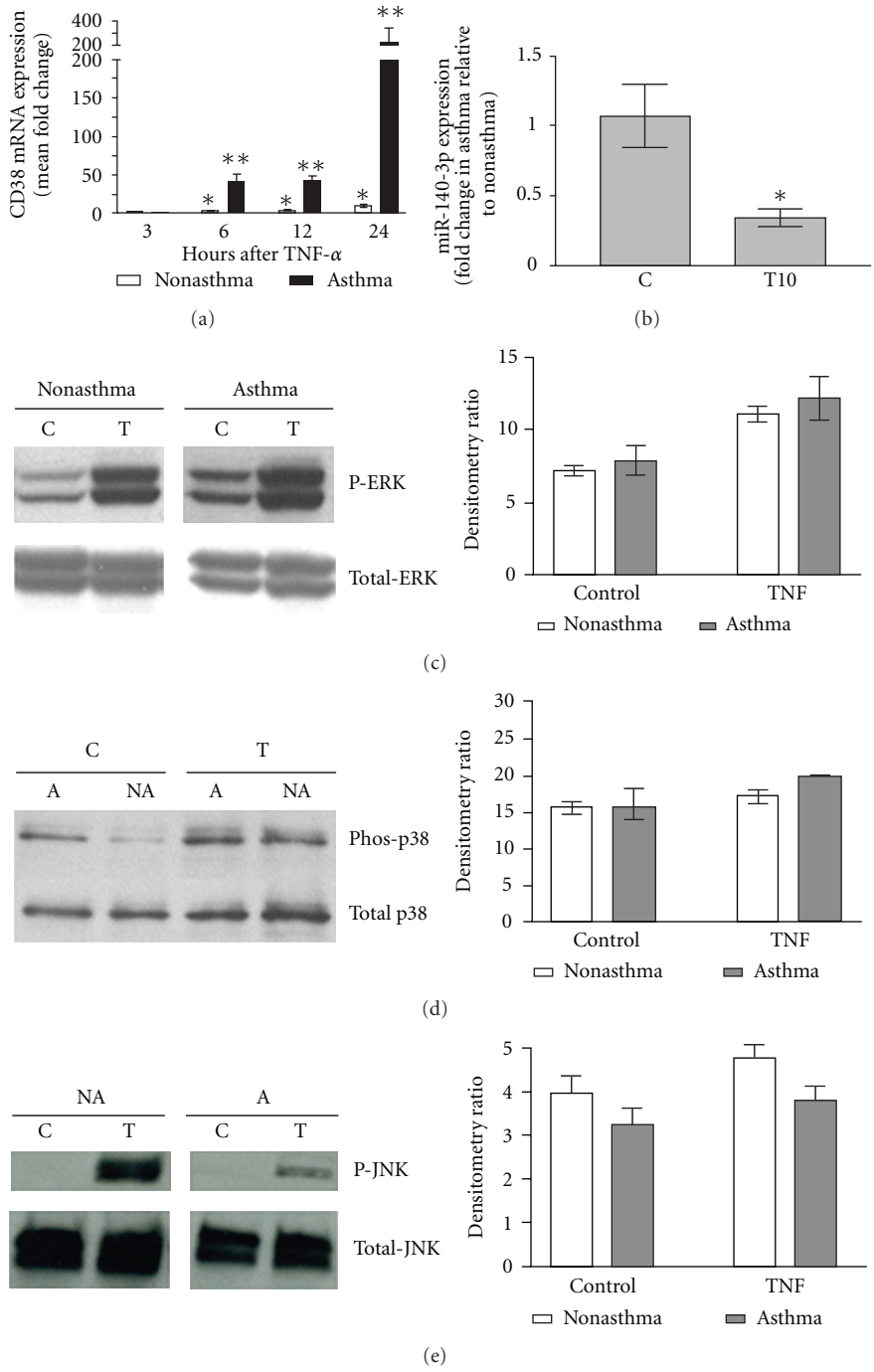

Figure 1: Altered signaling mechanisms contributing to asthmatic airway smooth muscle cell phenotype. Studies in our laboratory revealed some important changes in the HASM cells from asthmatic donors compared to the cells from nonasthmatic donors, suggesting a potential role for these mechanisms in the asthmatic phenotype of ASM cells. (a) Inflammatory cytokine TNF-α (10 ng/mL) induced differentially elevated CD38 expression in asthmatic HASM cells compared to the nonasthmatic HASM cells, as early as 6 hrs following addition of the cytokine ($n = 3$/nonasthmatic or asthmatic HASM cells, $^{*}P < 0.05$, significantly different from the vehicle-treated controls; $^{**}P < 0.05$, significantly different from the nonasthmatic HASM cells treated with TNF-α). (b) In HASM cells from asthmatic donors, TNF-α (10 ng/mL, 24 hrs of exposure) caused significant attenuation of miR-140-3p expression compared to the nonasthmatic HASM cells. The basal miR-140-3p expression levels were comparable between nonasthmatic and asthmatic HASM cells ($n = 5$/nonasthmatic group; $n = 6$/asthmatic group, $^{*}P < 0.05$, significantly different from the nonasthmatic group). (c–e) (Left and right panels) HASM cells obtained from asthmatic donors showed elevated basal and TNF-α-induced activations of ERK and p38 MAP kinases compared to the nonasthmatic HASM cells. TNF-α-induced JNK MAP Kinase activation was attenuated in asthmatic HASM cells compared to the nonasthmatic cells (blots representative of 5 independent experiments). (a) and (c–e) are reproduced with permission from [12]. Panel (b) is Reproduced with permission from [28]. Altered signaling mechanisms contributing to asthmatic airway smooth muscle cell phenotype.

and ERK activation (phosphorylated forms), but not that of JNK as compared to cells from nonasthmatic donors (Figure 1) [12]. Activation of NF-κB and AP-1 was measured by analyzing their nuclear content as well as their binding to consensus sequences. Both these parameters were comparable in cells from asthmatics and nonasthmatics [12]. These results support the concept that asthmatic ASM cells are intrinsically programmed to express greater levels of MAP kinase activation and the greater induction of CD38 expression by TNF-α may involve increased rate of transcription rather than transcript stability. This conclusion is further supported by the fact that transcript stability was comparable in cells from asthmatics and nonasthmatics [12]. However, we cannot rule out the contribution of other transcription factors to the observed differential response of asthmatic ASM cells. Furthermore, signaling mechanisms independent of MAP kinases may also be involved in this differential induction. It should be noted that significant ERK and p38 phosphorylation were reported in both epithelial cells and smooth muscle cells obtained from severe asthmatics, suggesting that these MAP kinase pathways may have an important role in the pathogenesis of severe asthma [31–33]. The levels of expression of kinases upstream of ERK as well as the MAP kinase phosphatase-1 are comparable in cells from asthmatics and nonasthmatics [12]. Precisely how a higher level of activation of these MAP kinases is maintained in the asthmatic ASM cells is not entirely clear.

With respect to other signaling pathways involved in the regulation of CD38 and their potential contribution to the asthmatic phenotype, we explored the role of PI3 kinases in such regulation. We found that ASM cells from both asthmatics and nonasthmatics express comparable levels of class I PI3 kinase P110 isoforms α, β, and δ [34]. Inhibition of the α and δ isoforms by siRNA transfection causes comparable magnitude of inhibition of TNF-α-induced CD38 expression in cells from asthmatics and nonasthmatics [34]. However, under comparable levels of siRNA-mediated downregulation of expression, the residual CD38 expression in asthmatic ASM cells was significantly higher than expression in cells from nonasthmatics [34]. Inhibition of the β isoform has no effect on CD38 expression. These results, while demonstrating similar expression levels of class I PI3 kinase isoforms in asthmatic and nonasthmatic ASM cells, show decreased sensitivity of CD38 expression in asthmatic ASM cells to inhibition of PI3 kinases. This is yet another example of an asthmatic phenotype of airway smooth muscle cells in terms of differential contribution of signaling pathways to the regulation of expression of specific genes. In this context, studies have shown that PI3 kinase signaling contributes to the proliferative response of ASM cells from asthmatics, while the ERK MAP kinase pathway seems to be involved in cells from nonasthmatic donors [32].

5. Posttranscriptional Regulation

Posttranscriptional regulation of genes is a mechanism aimed at altering the target gene expression swiftly in response to an environmental cue. In light of recent advancements in understanding microRNA regulation of gene expression, it appears that posttranscriptional regulation is also a fine tuning mechanism to delicately balance gene expression in cells (reviewed in [35]). Studies in our laboratory have shown that CD38 expression is regulated posttranscriptionally, at the level of transcript stability [29]. MAP kinases p38 and ERK1/2 regulate CD38 expression by modulating the transcript stability. The exact mechanism involved in the regulation of CD38 mRNA stability is not known. However, our studies focused on both RNA-binding proteins and microRNA as the potential mechanisms involved in the post-transcriptional regulation of CD38.

Specific short sequence motifs, primarily located on the 3′ untranslated region (3′UTR), interact with RNA-binding proteins to modulate the stability or translatability of the transcript [36, 37]. Adenylate-uridine- (AU-) rich motifs and cytosine-guanosine-uridine- (CGU-) rich motifs are the major sequence motifs found in 3′UTR of many transcripts and act as the *cis* elements responsible for mediating mRNA stability [38, 39]. The CD38 3′UTR possesses 4 AU-rich motifs, indicating a potential role for these *cis* elements in the post-transcriptional regulation of CD38 expression. *In vitro* pull down assays, using synthetic RNA oligonucleotide containing one of the AU-rich elements of CD38 3′UTR and HASM cell lysates, showed that the RNA-stabilizing protein, human protein R (HuR), the translational modulator protein and T-cells intracellular antigen-1 (TIA-1) bind selectively to the AU-rich element, in the presence of TNF-α. However, transient over expression of HuR in the HASM cells did not result in altered CD38 mRNA expression, suggesting RNA destabilizing proteins, such as tristetraprolin (TTP), may have a counteracting role in TNF-α-induced CD38 expression in HASM cells (unpublished results).

MicroRNAs (miRNA) are emerging as molecules with important roles in gene regulation in different orders of living organisms. Encoded from intronic or intergenic regions of the genome, miRNAs regulate the expression of a significant portion of human genes [40]. The general mechanism of regulation by miRNA is through modulating the stability or translatability of the target mRNA [35]. On average, a single miRNA can target and regulate ~100 different genes, although it is suggested that the cluster of genes regulated by a single miRNA would belong to a particular cellular function. Bioinformatic screening of CD38 3′UTR revealed predicted targets for several miRNAs. Systematic studies on the expression and role of some of these miRNAs have been carried out in our laboratory. Luciferase-CD38 3′UTR reporter assays confirmed that miR-140-3p, one of the miRNAs predicted to target CD38, functionally targets CD38 3′UTR [28]. Further studies using over expression of miR-140-3p mimic in HASM cells showed that CD38 mRNA and protein expressions were significantly downregulated by miR-140-3p [28]. Findings of our study suggest that the inhibitory effect of miR-140-3p mimic on CD38 expression is only partially mediated through direct binding to the CD38 3′UTR, since there was only a marginal inhibition of luciferase activity by miR-140-3p mimic. The dominant effect of miR-140-3p on CD38 expression appears to be mediated indirectly through transcriptional mechanisms,

Table 1: A partial list of miR-140-3p gene targets predicted by TargetScan and microRNA.org.

Receptors and channels	ARHGAP3, IRGQ, SCN3A, NKIRAS2, GPR12, GIT1, GABRB2, PLEKHA1, KCNA7, TRPM7, SGSM1, GPR158, REEP5, SGIP1, GAB2, RASGEF1B, FKBP1A, and RGS1
Cytokine/chemokine regulation	SOCS4, CDK6, MMP16, TGIF2, ADAM17, ITK, CHL1, ADAM9, CXCL11, IL8, IL6, and CXCL6
Related to transcriptional regulation	CREB1, SP3, TAF2, SP4, TCEB3, SIRT1, E2F7, KLF4, RAB2A, FOX2, NFYA, NKRF, HDAC4, GABPB1, SP3, KLF5, IKB, and KB
MAPK and PKC/MTOR pathways	PTEN, RAP1B, RPS6KA3, RICTOR, MAPK1, PPFIBP1, CDS2, MARCKS, MAP2K6, and IL24,
Related to actin and myosin	MYLK4, MAPRE3
Related to proliferation	ZNF3

by inhibiting activation of p38 MAP Kinase and NF-κB. MicroRNA target prediction algorithms revealed an array of genes potentially targeted by miR-140-3p in humans (Table 1). We also found that miR-140-3p expression was significantly attenuated by TNF-α exposure in asthmatic HASM cells compared to nonasthmatic HASM cells [28]. Whether the greater degree of downregulation of miR-140-3p in asthmatic ASM cells compared to ASM cells from nonasthmatics contributes to the reported differential induction of CD38 expression in these cells is not clear, but remains an attractive hypothesis. These findings also suggest that CD38 and miR-140-3p may be part of a differential gene expression profile in asthmatic HASM cells, indicating a potential role for these two molecules in the pathogenesis of asthma. Recent investigations have provided insights into the role of microRNAs in the regulation of ASM cell phenotype and ASM contractility [41, 42]. Studies have also reported altered microRNA expression profiles in various cells obtained from asthmatic donors [43, 44]. The roles of specific microRNAs in the development of AHR have also been demonstrated by studies in mouse models of asthma [45–47]. MicroRNA target prediction algorithms revealed an array of genes potentially targeted by miR-140-3p in humans (Table 1). The table has a partial list of the genes that encode proteins associated with G-protein-coupled receptor function, ion channels, chemokines, transcription factors, signaling proteins, contractile and cytoskeletal elements, and cell proliferation. It should be noted that some of the genes targeted by the microRNA are involved in the regulation of CD38 expression, that is, transcription factors, MAP kinases, and PKC signaling-associated proteins. Downregulation of expression of the chemokines by miR-140-3p has the potential to have significant anti-inflammatory effects.

6. Conclusions

Our investigations of CD38 expression have revealed some important differences between ASM cells from asthmatics and nonasthmatics in the sensitivity to inflammatory cytokines in terms of level of expression, sensitivity of expression to inhibition of signaling intermediates such as MAP Kinases and PI3 Kinases, in microRNA expression, and post-transcriptional regulation of expression. These differences in the asthmatic ASM cells are maintained over at least 5 passages in culture. Studies in cytokine or allergen-induced inflammatory airway disease mouse models have revealed that CD38 contributes to airway hyperresponsiveness both through its role in generating calcium-mobilizing second messenger molecules and thereby the contractility of airway smooth muscle and through its role in adaptive immune response. We propose that increased capacity for CD38/cADPR signaling in airway smooth muscle in asthma contributes to the development of airway hyperresponsiveness. Whether the differences that we report transcend all asthma phenotypes is not currently known and requires further investigation.

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Review Article

Neuronal Modulation of Airway and Vascular Tone and Their Influence on Nonspecific Airways Responsiveness in Asthma

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The autonomic nervous system provides both cholinergic and noncholinergic neural inputs to end organs within the airways, which includes the airway and vascular smooth muscle. Heightened responsiveness of the airways to bronchoconstrictive agents is a hallmark feature of reactive airways diseases. The mechanisms underpinning airways hyperreactivity still largely remain unresolved. In this paper we summarize the substantial body of evidence that implicates dysfunction of the autonomic nerves that innervate smooth muscle in the airways and associated vasculature as a prominent cause of airways hyperresponsiveness in asthma.

1. Introduction

With the exception of airway smooth muscle, perhaps no other group of cells has as clear a role in the pathogenesis of asthma as the neurons comprising the afferent and efferent innervation of the airways and lungs. The symptoms of asthma—wheezing, dyspnea, chest tightness, cough, reversible airways obstruction, mucus hypersecretion, and airways hyperresponsiveness—all inextricably link the nervous system to this disease. It is thus remarkable that in the 440 pages of the National Heart, Lung and Blood Institute (NHLBI) guidelines on asthma, nerves are mentioned in just one sentence [1]. Nerves are not mentioned at all in the British Thoracic Society (BTS) guidelines for asthma [2]. Even the recent and potentially landmark study by Peters et al. [3], in which the anticholinergic tiotropium was found to be at least as good as steroids or β-agonists (perhaps better) for treatment of asthma, nerves are not mentioned in the article itself nor in the accompanying editorial [4]. In this brief review we summarize the large body of evidence supporting a primary role for airway autonomic nerve dysfunction in the hyperresponsiveness of the airway smooth muscle in asthma.

2. The Understated Role of Nerves in Asthma

Guidelines such as those produced by the NHLBI and BTS, in which immune cells including eosinophils are given a central role in asthma pathogenesis appropriately highlight the prominent feature of inflammation in the asthmatic lung. Inflammation may precipitate airways hyperresponsiveness [1, 5–8]. But the association between inflammation and airways hyperresponsiveness has probably been overemphasized [9–13]. The bias towards inflammation in asthma guidelines reveals the disproportionate influence immunologists, and allergists have had overdefining this disease for national and international medical organizations as well as their influence over the direction of asthma-related research. As asthma prevalence and asthma mortality rates have remained largely unchanged in the decades where inflammation has become a central theme in asthma research and therapy, it may be time for a new perspective on old concepts of the pathogenesis of reactive airways disease.

Given the strong case for neural dysfunction in asthma, it is surprising how little attention airway nerves receive in the published literature. The under emphasis on nerves in asthma and the exaggerated influence of inflammation in

asthma can be illustrated by comparing the scant references to neural mechanisms in this disease with the incessant discussions of eosinophils in asthma guidelines and in all of asthma-related literature. This is especially surprising, given how strong the evidence is in favor of neural mechanisms in asthma and how comparatively weak the evidence is supporting a role for eosinophils in this disease. Even the most ardent proponents might struggle to make a strong case for a role of eosinophils in asthma. There is no increase in the risk of asthma for patients with hypereosinophilic syndrome [14]. Nonasthmatic atopic patients develop a profound eosinophilia of the airways upon exposure to allergen but develop few if any of the symptoms of asthma [15, 16]. Many asthmatics have eosinophil levels in their airways and air spaces that are comparable to that of nonasthmatics [5, 6, 17]. Experimental therapies such as anti-IL-5 and recombinant IL-12 have a profound effect on circulating and airway eosinophil numbers and on allergen-induced recruitment of eosinophils to the airways but little or no effect on asthma symptoms in most patients and no effect on airways reactivity [18–21]. Even steroids, which markedly inhibit eosinophil function, survival and recruitment to the airways, have only modest effects on airways hyperresponsiveness [22]. And yet, in spite of what seems to be a clear role for the nervous system in asthma and at best a debatable role for eosinophils, nerves are mentioned in one sentence combined in the NHLBI and BTS guidelines while these same guidelines, cite eosinophils 85 and 43 times, respectively. This imbalance is pervasive in the published literature as well. Since 2001, several years after Leckie and colleagues reported their disappointing results with anti-IL-5, there have been over 4600 papers published with the keywords of "asthma" and "eosinophil" but less than 450 papers with the keywords "asthma" and "nerve". Indeed, there have been more papers published with the keywords "eotaxin" and "asthma" over the past 10 years than papers with the keywords "asthma" or "COPD" and "nerves" combined.

The mechanisms of airways hyperresponsiveness are poorly understood. It seems all but certain that smooth muscle is central to regulating airways reactivity. However, studies of airways smooth muscle contractility *in vitro*, conducted using airways obtained from asthmatic and nonasthmatic lung donors, yield results that are somewhat varied [23–28]. Thus, an argument can be made that neither smooth muscle contractility (efficacy) nor responsiveness (potency) differs to any great extent between airways obtained from diseased or nondiseased patients. Bronchodilators are also largely equally effective in smooth muscle from asthmatics and nonasthmatics [29, 30]. The defect in asthma may therefore manifest only within the context of the intact body and lung.

If, however, we accept the evidence in support of the hypothesis that airway smooth muscle accounts in large part for the most defining pathophysiological features of asthma (reversible airways obstruction and airways hyperresponsiveness) it is then critical to determine what ultimately regulates airway smooth muscle contraction. Airway smooth muscle generates little myogenic tone and so contraction depends upon the actions of contractile agonists. Despite an extensive list of autacoids and neurotransmitters that *can* contract human airway smooth muscle, a survey of the published literature suggests that only 3 endogenously released ligands, acetylcholine, histamine, and the cysteinyl-leukotrienes, reliably contract human airway smooth muscle to any significant extent and in physiologically relevant conditions in the airways of asthmatics. What so clearly defines the role of the nervous system in regulating the airways hyperresponsiveness in asthma is the indisputable source of the acetylcholine that regulates airway smooth muscle tone and the profound effects of anticholinergics on the airways obstruction and airways reactivity that define this disease.

3. Autonomic Innervation of Human Airway Smooth Muscle

The autonomic nervous system plays a primary role in regulating airway smooth muscle tone. The highly regulated activity of these nerves allows ongoing input to the airway smooth muscle such that basal tone is regulated on a breath by breath basis. The origin of this ongoing drive depends upon centrally (i.e., brainstem) mediated activity established by both respiratory and reflexive inputs [31–34]. In most animals and in humans, stimulation of airway autonomic nerves evokes near maximal constrictions of the airways through the actions of acetylcholine released from postganglionic parasympathetic nerves. Alternatively, activation of airway autonomic nerves can reverse completely spasmogen-evoked bronchoconstriction through the actions of noncholinergic neurotransmitters such as nitric oxide (NO) and vasoactive intestinal peptide (VIP) and related peptides. It follows logically that dysfunction or dysregulation of airway autonomic nerves is likely to contribute to the pathogenesis of asthma and COPD (reviewed in [35]).

For years it had been widely assumed that noncholinergic neurotransmitters mediating relaxations of the airways were coreleased with acetylcholine from a single population of postganglionic parasympathetic nerves. It was further speculated that these noncholinergic cotransmitters served as a brake on the parasympathetic nervous system, preventing excessive constriction during periods of elevated autonomic tone. Our studies have revealed, however, that anatomically and physiologically distinct parasympathetic nerves mediate cholinergic contractions and noncholinergic (nitrergic) relaxations of the airways [36–38]. Importantly, reflexes differentially regulate these distinct parasympathetic pathways [34, 39]. The existence of two parasympathetic pathways with opposing actions on the bronchial musculature changes entirely how autonomic nerve-dependent regulation of airway caliber should be viewed. Bronchospasm could be evoked by increases in cholinergic nerve activity or withdrawal of nitrergic neural activity. Conversely, increased nitrergic nerve activity or decreased cholinergic tone could elicit bronchodilatation. The role of the autonomic nervous system in disease must also now be viewed differently. With distinct neuronal pathways mediating contractions and relaxations of airway smooth muscle, dysfunction or dysregulation of either parasympathetic pathway could account for

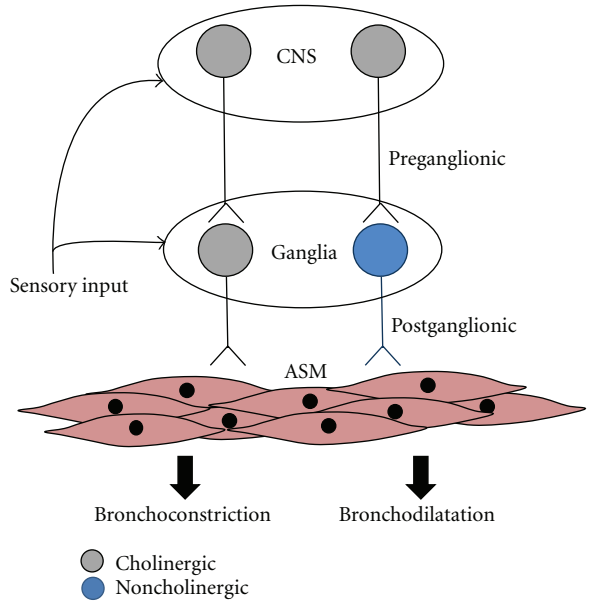

Figure 1: Two distinct vagal parasympathetic pathways regulate airway tone. Cholinergic preganglionic neurons originate in the brainstem and provide cholinergic drive to airway autonomic ganglia. Cholinergic postganglionic neurons are the major contractile input to the airways, whereas noncholinergic neurons expressing nitric oxide and vasoactive intestinal peptide provide the relaxant innervation to the airways. Airway sensory nerves contribute differential reflex regulation over cholinergic and non-cholinergic vagal pathways at the level of the brainstem and/or the airway ganglia. Dysfunction in ganglionic neurotransmission, neuromuscular transmission, or sensory reflexive control will precipitate changes in airway smooth muscle reactivity.

the alterations in airway tone associated with asthma and COPD (Figure 1).

4. Autonomic Dysfunction and Asthma

There is indisputable evidence supporting the hypothesis that dysregulation of airway cholinergic nerves contributes to the pathogenesis of airways obstruction and airways hyperresponsiveness. Cholinergic nerve-mediated obstruction of the airways is increased in asthma and COPD [28, 40]. Airways hyperresponsiveness is also associated with alterations in cholinergic nerve function. Anticholinergics markedly reduce (10–20-fold) or abolish airways reactivity to a wide variety of spasmogens and stimuli including prostanoids, histamine, bradykinin, capsaicin, hyperpnea, exercises and allergen (reviewed in [35]; Table 1). Airways hyperresponsiveness associated with extrapulmonary disorders may also be dependent upon alterations in airway autonomic control. Bronchospasm initiated by gastroesophageal reflux or airways obstruction associated with allergic rhinitis is prevented by anticholinergics. Similarly, in patients with upper respiratory tract infections, the marked increases in airways reactivity precipitated by the infection are reversed by atropine [41]. More recent studies suggest anticholinergics might be highly effective in treating asthma. Soon after the study of Peters et al. [3], which suggested that tiotropium was superior to steroids and β-agonists in controlling asthmatic airway function, 2 subsequent studies reported similar findings when using the ultra-potent and long acting anticholinergic [42, 43]. The reported effects of tiotropium on airway smooth muscle mass suggest that in addition to relieving functional obstruction, anticholinergics may play an important role in reversing airways remodeling [44].

Table 1: Effect of anticholinergics on airways hyperresponsiveness in asthma[a].

Provocation	Effect
Beta blockers	Abolished response
Bradykinin	5-fold increase in PD_{35}
Capsaicin	60% reduction in response
Distilled water	50–100% reduction in response
Exercise	30% reduction in response
Histamine	10-fold increase in PC_{100} SRaw
Hyperpnea	Abolished response in children
Prostaglandin D_2	12- to 22-fold increase in PC_{20}
Psychogenic stimulation	Abolished response
Reflux or esophageal acidification	Abolished response
Thromboxane A_2	23-fold increase in PC_{20}

[a] Anticholinergics used were either ipratropium bromide or atropine delivered via aerosol. Results reviewed in detail elsewhere [35]. Abbreviations: PC_{20} and PD_{35}: provocative concentration (or dose) of agonist producing a 20% or 35% decrease, respectively, in forced expiratory volume in 1 sec (FEV_1); PC_{100} SRaw: provocative concentration of agonist producing a 100% increase in specific airways resistance.

Evidence suggesting that nitrergic parasympathetic nerves are dysfunctional in airways disease is circumstantial

but compelling. In humans and in many animal species, adrenergic nerves are sparse or absent in the airways and without apparent influence over airway smooth muscle tone [45]. Consequently, nitrergic parasympathetic nerves represent the only functional relaxant innervation of airway smooth muscle. Importantly, in asthma, an inability to dilate with deep inspiration and not excessive smooth muscle constriction may underlie the pathogenesis of airways hyperresponsiveness [46]. In a preliminary report, inhibitors of NO synthase (NOS), which can inhibit relaxations mediated by airway nitrergic parasympathetic nerves, prevent the bronchoprotective effects of deep inspiration in normal patients [47]. Perhaps airway nitrergic nerves regulate airways reactivity by counteracting the actions of spasmogens through tonic, ongoing effects in the airways or by subserving a compensatory role with increased activity following challenge. Consistent with these hypotheses, NO synthase inhibitors exacerbate airways responsiveness to bradykinin in mild asthmatics, a compensatory mechanism that is lost in severe asthmatics [48]. Although the source of the nitric oxide was not determined in these clinical studies, experiments using animals and studies of human airway preparations indicate that parasympathetic nerves are one potential source [33, 49–52]. Pathological and molecular biological studies are also consistent with the hypothesis that dysregulation of airway noncholinergic nerves contributes to the pathogenesis of asthma and COPD. For example, arginase (which competes with neuronal NOS for the substrate L-arginine) activity is increased in models of asthma, thereby leading to a reduced capacity to produce neuronal NO [53]. Mutations in the gene encoding the neuronal isoform of NOS have been associated with asthma [54, 55]. These mutations are associated with a decrease in exhaled nitric oxide in asthma [56]. In fatal asthma, VIP-containing nerves have been reported to be sparse in the airways [57]. VIP and NOS are colocalized to airway ganglia [51]. All of these observations indicate that dysregulation of nitrergic parasympathetic nerves might contribute to the pathogenesis of airways diseases.

5. Autonomic Regulation of Vascular Tone in Asthma

Vascular beds in the airways play an important role in basal airway obstruction through the regulation of airway wall volume [58]. Mucosal edema is a prominent feature in the asthmatic airways, and this contributes significantly to airflow limitations [59]. The airway vasculature, however, can also directly modulate airway smooth muscle reactivity by regulating the clearance of bronchoactive agents from the airway wall. For example, animal studies have shown that vasoconstriction or reduced vascular perfusion of the airways significantly potentiates airway smooth muscle responsiveness to a variety of bronchospastic agents [60–62] (Figure 2). In asthmatics, intravenous angiotensin II increases methacholine bronchoconstriction but does not alter bronchoconstriction evoked by endothelin, an autacoid that constricts airway vascular smooth muscle (while

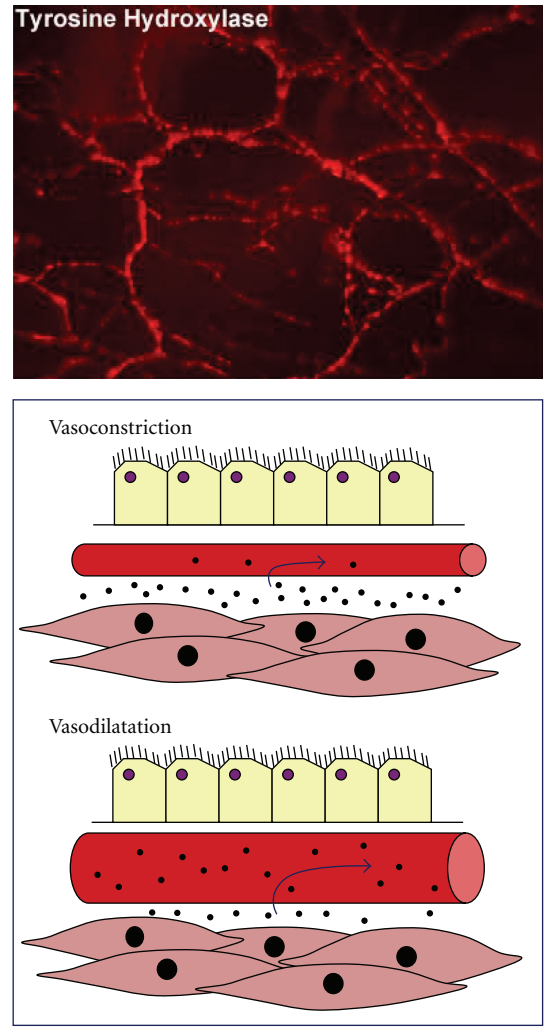

Figure 2: Airway vascular tone and blood flow regulate airway smooth muscle reactivity. The airway vasculature is densely innervated by sympathetic (tyrosine hydroxylase expressing) neurons which provide a basal level of adrenergic vascular tone. Soluble and insoluble particles in the airway wall are actively cleared by the submucosal vasculature. Increased blood flow is associated with increased clearance, and this can significantly modify airway smooth muscle reactivity to bronchoactive agents which are deposited onto, or generated within, the airway wall. See text for additional details.

methacholine relaxes vascular smooth muscle; [63, 64]). The loop diuretic furosemide also reduces airways reactivity to exogenous stimuli in humans [27], perhaps via a vasodilatory action since it does not relax airway smooth muscle *in vitro*. Similarly, *in vivo* epinephrine (a nonselective adrenergic agonist that would evoke both bronchodilatation and vasoconstriction) has no effect on reactivity yet *in vitro* (where the vasculature is no longer intact), it is more potent and efficacious than the β-adrenergic agonist albuterol at preventing airway smooth muscle constriction [65].

As is the case with airway smooth muscle, vascular smooth muscle possesses a baseline level of tone that is dependent upon ongoing activity of the autonomic nervous

system [61]. However, unlike the airway smooth muscle, vascular tone is heavily dependent upon adrenergic sympathetic nerves acting via alpha-adrenergic receptors (reviewed in [66]). Neuropeptide Y also constricts the vascular smooth muscle secondary to sympathetic nerve activation, whereas activation of parasympathetic nerves evokes vasodilatation following the release of acetylcholine or nitric oxide and vasoactive intestinal peptide. In some species neuropeptide expressing sensory nerves can mediate vasodilatation via axon reflexes, although this is not likely a prominent mechanism of vasoregulation in humans.

6. Mechanisms of Autonomic Dysfunction

While it is clear that the nervous system is essential to the reactivity of the airways in asthma, it is unclear precisely what drives dysfunction of airway nerves in asthma. The simplest explanation might be that inflammation alters airway autonomic function in asthma. Airways inflammation has been associated with enhanced cholinergic responses following altered prejunctional control mechanisms (such as the muscarinic M2 autoreceptor that normally prevents acetylcholine release from nerves) or by sensitizing neurotransmission through the parasympathetic autonomic ganglia (the synaptic relay between pre- and postganglionic neurons) (reviewed in [35]). Nitrergic relaxant nerve responses may also be diminished in the asthmatic airways. The synthesis or degradation of the peptidergic and nitrergic neurotransmitters (or their substrates) utilized by nitrergic nerves may be perturbed in asthma via the actions of peptidases, free radical scavengers, and arginase. An alternative explanation is that the effects of airway inflammation are indirectly linked to autonomic nerve dysfunction. For example, sensitization and altered activity of airway sensory nerves is a common feature in asthma. Sensory nerves provide direct inputs to airway autonomic pathways, both at the level of the brainstem and the autonomic ganglia, and it is therefore likely that altered sensory function contributes to changes in autonomic drive to the airways [35].

7. Conclusions and Future Directions

It seems possible that an overemphasis on the role of inflammation in models of asthma, and less attention to the central role of airways hyperresponsiveness, has contributed to the frequent failure to translate promising therapeutic strategies discovered in animals into patients with asthma [67, 68]. Important insights into the mechanisms of inflammation in asthma have been established. In clinical studies, both leukotrienes and IL-5 induce pulmonary eosinophilia [69, 70], whereas in asthmatics, leukotriene modifiers and anti-IL-5 reduce eosinophil and basophil recruitment to the airways [19, 20, 28, 71]. The Th2 cytokines IL-4 and IL-13 also seem to play a role in asthmatic inflammation [1], but therapies targeting IL-5 [19–21], IL-4 [72], or IL-13 [73, 74] have provided little or no relief of asthma symptoms and little relief from airways hyperresponsiveness. By contrast, anticholinergics have proven remarkably effective at reducing the acute responses to allergen challenge and consistently decrease airways obstruction and airways reactivity in asthmatics. There is much unknown about the innervation of the airways. Given its central role in the pathogenesis of asthma, efforts to fill the many gaps in our understanding of airway neural control are warranted.

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Review Article

A Brief History of Airway Smooth Muscle's Role in Airway Hyperresponsiveness

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A link between airway smooth muscle (ASM) and airway hyperresponsiveness (AHR) in asthma was first postulated in the midnineteenth century, and the suspected link has garnered ever increasing interest over the years. AHR is characterized by excessive narrowing of airways in response to nonspecific stimuli, and it is the ASM that drives this narrowing. The stimuli that can be used to demonstrate AHR vary widely, as do the potential mechanisms by which phenotypic changes in ASM or nonmuscle factors can contribute to AHR. In this paper, we review the history of research on airway smooth muscle's role in airway hyperresponsiveness. This research has ranged from analyzing the quantity of ASM in the airways to testing for alterations in the plastic behavior of smooth muscle, which distinguishes it from skeletal and cardiac muscles. This long history of research and the continued interest in this topic mean that the precise role of ASM in airway responsiveness remains elusive, which makes it a pertinent topic for this collection of articles.

1. Introduction

In this paper we review the history of the link between airway smooth muscle (ASM) and the phenomenon of bronchial hyperreactivity or hyperresponsiveness (BHR) which is a defining feature of asthma. Figure 1 shows the number of PubMed citations generated by a search for "airway smooth muscle + airway hyperresponsiveness + asthma." These results suggest that there is an increasing interest in the role of ASM in AHR, but what is the history of evidence to support a link?

It has long been recognized that muscular constriction of the bronchi contributes to airway narrowing in asthma. In his 1698 treatise on asthma Floyer wrote, "the Bronchia are contracted … and that produces the Wheezing noise in Expiration, and that this Symptom does not depend on Phlegm is plain, because the Hysteric, who have no Phlegm, Wheeze very much" [1]. In mid-nineteenth century, Salter [2] wished that it could be "shown beyond cavil that spasmodic stricture of the bronchial tubes is the only possible cause of asthma, that it is adequate to the production of all the phenomena." He was referring to the "spastic contraction of the fiber-cells of organic muscle," which we now refer to as the airway smooth muscle (ASM).

In a landmark study of the pathology of asthma Huber, and Koessler [3] described and quantified the increased mass of ASM. The accumulated evidence for an increase in muscle mass and the relative contributions of hypertrophy and hyperplasia to this process has been recently summarized [4]. Thus there is little doubt that ASM is increased in asthma. The questions that remain are whether this increase is the cause of airway hyperresponsiveness (AHR) or whether there are additional fundamental changes in the phenotype of the muscle which contribute to AHR.

Bronchial responsiveness in asthmatics was first reported by Alexander and Paddock in 1921 [5] when they noted that an attack could be precipitated by subcutaneous injections of pilocarpine. Similarly Weiss et al. [6] found that asthmatics became more breathless and had a greater fall in vital capacity in response to intravenous histamine than did non-asthmatic subjects. Subsequent early studies confirmed that asthmatics responded excessively to a wide variety of stimuli including acetyl-beta-methylcholine [7], carbachol [8], histamine [9], slow reacting substance of anaphylaxis

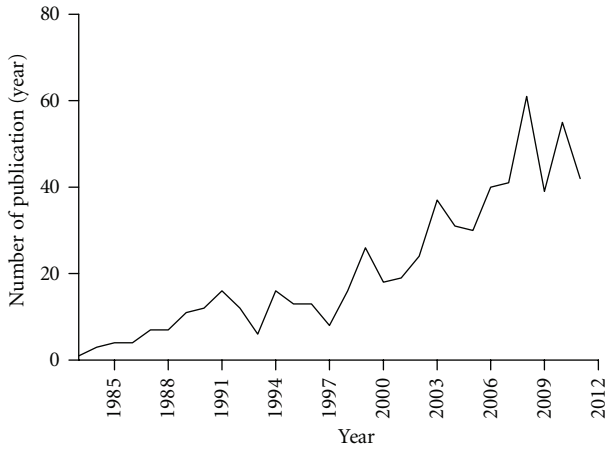

Figure 1: PuMed search for (ASM + AHR + asthma).

[10], prostaglandin F2α [11], propranolol [12], cold air [13], sulphur dioxide [14], dust [15], and exercise [16]. Most of these act directly on smooth muscle but others act to cause secondary release of contractile agonists (e.g. cold air) or inhibition of bronchodilating factors (e.g. propanolol). In more recent literature these methods of eliciting an airway response have been termed *direct* (via ASM) and *indirect* (via release of inflammatory mediators and subsequent ASM activation) measures of airway responsiveness.

These studies illustrated a key feature of AHR, that it is nonspecific. If a subject is hyperresponsive to one stimulus they are hyperrresponsive to all agents that act by stimulating smooth muscle contraction. This observation was important since it suggested that the phenomenon was primarily postjunctional (i.e., on the muscle side of the neuromuscular junction) and not related to specific abnormalities of any specific agonist receptors on smooth muscle cells). This supports the hypothesis that a change in ASM phenotype is responsible for the phenomenon of AHR in asthma but an additional important early observation was that AHR was not limited to asthmatics. Patients with a variety of diseases characterized by airway obstruction show AHR and the degree of airway responsiveness is related to the degree of baseline airway obstruction [17–19]. These results suggest that the responsiveness may be consequence of the airway narrowing rather than a predisposing factor. However in asthma, AHR is relatively independent of baseline lung function [20] suggesting that the underlying mechanisms may be distinct from the AHR seen in COPD and other airway diseases.

A final seminal early observation was that there were variations in airway responsiveness over time. De Vries et al. showed that there was diurnal variation in responsiveness [21] with the greatest responsiveness occurring at night when the baseline airway narrowing tends to be greatest. Kerrebijin (1970) showed that AHR increases after an acute spontaneous attack of asthma and then improves over time as the attack subsides [22], again suggesting that AHR is a consequence of asthma, or at least that a portion of the AHR was variable and unlikely to represent a fundamental phenotypic change in the muscle. Parker et al. showed that AHR occurred during or after a respiratory tract infection in normal subjects supporting the concept of acquired, reversible AHR [18]. Additional important observations were that AHR increased after the late, but not the early, asthmatic response [23] to inhaled allergen and that AHR could be attenuated by prolonged anti-inflammatory therapy [24].

2. Airway Smooth Muscle and Airway Responsiveness

Despite the increasing interest in AHR during the 60s and 70s, there were few attempts to study the mechanism. As early as 1951, Schild et al. [25] found that lung tissue and bronchial muscle obtained from an asthmatic patient released more histamine and responded with contraction to challenge with house dust or pollen compared to a non-asthmatic, but it was not until the early 1980s that there was speculation that AHR was caused by an intrinsic alteration in ASM structure or function. The prevalent theories prior to that where related to pre-existing airway narrowing [26, 27], increased sensitivity of airway irritant receptors [28] or a relative deficiency of beta adrenergic bronchodilation [29]. Freedman [30] and Benson [26] were among the first to systematically consider the potential link between the structural and functional changes in the airways and AHR. They pointed out that airway wall thickening and/or baseline airway smooth muscle tone could amplify the airway narrowing caused by a subsequent stimulus supporting the concept that AHR was a manifestation of airway disease not a root cause.

A pivotal study by Woolcock et al. [31] published in 1984 showed that an important feature of AHR in asthma was an increase in maximal achievable airway narrowing in response to histamine; most nonasthmatic subjects can inhale high concentrations without much airway narrowing. They showed that asthmatics not only show a response at a much lower dose or concentration than nonasthmatics (increased sensitivity) but that the amount of airway narrowing measured by a decline in forced expiratory flow is much greater. This was attributed to a lack in asthmatics of a normal mechanism that inhibits severe airway narrowing in nonasthmatics and they hinted at a link to maximal ASM contraction.

Studies of excised human airway smooth muscle began in the 1980s and for the most part failed to incriminate ASM, although most of the initial studies examined only isometric force. Although some studies suggested that ASM from asthmatics was stronger [32, 33] the bulk of the data [34–38] show that the maximal force that ASM can generate does not differ in asthmatic and nonasthmatic individuals. These studies spawned a number of different avenues of investigation in an attempt to explain AHR. Generally these studies focused on additional properties of ASM that could be important in generating AHR or on additional explanations for AHR that did not involve a fundamental change in ASM phenotype. In 1986, Moreno et al. [39] presented an extensive theoretical analysis of the geometric factors which could link ASM activation and excessive airway

narrowing, amplifying the earlier work of Freedman [30]. James et al. [40] and Wiggs et al. [41] quantified the potential contribution of airway wall remodeling to increased maximal airway narrowing. Lambert et al. [42] concluded that the increase in smooth muscle mass was potentially the most important structural change to explain AHR (provided that the increased muscle mass retained its contractile phenotype). This conclusion has been supported by recent work from Oliver et al. [43] who added cyclical stress to the model to simulate breathing. Their analyses confirmed the importance of increased muscle mass and also suggested that increased muscle could explain the failure of asthmatics to respond to deep inspirations.

The other mechanical properties of ASM that have been explored as potential contributors to AHR include an increased maximal amount of shortening, increased velocity of shortening, reduced relaxation, and a reduced effect of strain on the reduction of force that occurs with breathing and deep inspiration. Ma et al. [44] examined the maximal shortening and the shortening velocity of primary isolated ASM cells from asthmatic and normal subjects and found both greater maximal shortening and faster shortening associated with an increased expression of myosin light chain kinase. Léguillette et al. [45] studied the relative expression of two isoforms of human myosin in the ASM of asthmatic and nonasthmatic subjects. They found that there was increased mRNA for the SM-B isoform in asthmatic tissue. SM-B contains a 7 amino acid insert and can be shown to propel actin faster than the SM-A isoform. These data suggest that a change in the relative proportion of the two myosin isoforms could increase ASM shorting velocity and could increase AHR in asthmatics. They did not measure the relative abundance of the protein for the two isoforms.

3. Airway Smooth Muscle Adaptability

A whole new area of investigation was heralded in 1995 by the publication by Pratusevich et al. [46] showing that unlike skeletal muscle the length tension relationship of smooth muscle is plastic; the length at which maximal force and shortening occur can change dependent on the length history of the smooth muscle. This observation coupled with an important paper by Skloot et al. [47], also published in 1995, showing that in asthmatics deep inspiration (DI) fails to prevent airway narrowing, suggested a whole new paradigm, the possibility that there might be a fundamental difference in the ASM's response to stress or strain in asthma. A large number of *in vitro* and *in vivo* studies designed to establish the mechanism of this difference followed these publications. Although the physiological processes responsible for the beneficial effects of deep inspiration (DI) are unknown, they are thought to involve mechanical stretch of the ASM during lung inflation [48]. However other factors may also be involved including neural and humoral pathways [49].

3.1. Acute Length Perturbations. *In vitro* studies showed that the contractile capability of an isolated ASM strip is attenuated by subjecting it to length oscillations [50, 51]. Fredberg et al. [52] developed a model to demonstrate that mechanical strains in ASM caused by tidal breathing or DI causes detachment of myosin heads from actin sooner than it would during isometric contraction, leading to a steady-state equilibrium. They suggested that in asthma, disruption of this equilibrium leads to the "frozen" contractile state where the muscle is not stretched enough to allow enough mechanical perturbation to disrupt the cross-bridges.

DI is an inhalation that expands the lung volume toward total lung capacity. There are considerable data showing that DIs are effective in reversing bronchoconstriction in healthy subjects using measurements of resistance (Raw) and forced expiratory volume in one second (FEV_1). By contrast, DI is not effective or even further exaggerates existing bronchoconstriction in some asthmatic subjects, especially when the airway narrowing occurs during spontaneous or antigen-induced asthmatic attacks [53]. This paradoxical response to DI was recognized as early as the 1960s and 70s [28, 54]. There is a spectrum between the normal response (DI-induced bronchodilation) and severe asthma (DI-induced bronchoconstriction); mild and well controlled asthmatics behave more like nonasthmatics. However it was the observation that DI taken prior to a bronchoconstricting stimulus attenuates the subsequent airway narrowing that has rekindled major interest in this phenomenon [47, 55–61] which has been termed DI-induced bronchoprotection as opposed to bronchodilation. It has been suggested that asthmatics uniquely lack the bronchoprotective effect of DIs [60]. It has long been accepted that stretching contracted ASM by DI reduces bronchospasm by disrupting actin-myosin cross-bridges [52, 62]. However, when DI is taken prior to stimulation, there should be few or no cross-bridges. Hence bronchoprotection could not be explained by a physical detachment of cross-bridges. Wang et al. postulated that the bronchoprotective effect of DI can be explained by the adaptive behavior of ASM in response to DI [48, 51].

Length adaptation (also called plasticity) refers to the ability of the muscle to adapt its contractile capacity to length changes as mentioned above. Pratusevich et al. [46] showed that ASM is able to rapidly adapt to different lengths and maintain optimal force generation over a large length range. They observed that the adaptive process consists of two stages, an immediate reduction in force generation following the length change followed by a gradual recovery of the force toward that achieved before the length change. When length oscillation (to simulate DIs) was applied to unstimulated ASM, a similar two-staged adaptive process were observed [51]. A reduction in active force in response to stimulation was observed immediately after the oscillation and the magnitude of active force reduction was linearly related to the amplitude of the oscillation. After this initial reduction, the muscle undergoes the adaptation process by which active force increases gradually with each stimulation until stabilizing at the level prior to length oscillation. The adaptation process takes about 30 to 40 min to complete depending on animal species and how frequently the muscle is stimulated. McParland et al. [63] showed that ASM from pigs could adapt to a shortened state induced by carbachol

within 30 min, resulting in increased force, shortening and shortening velocity.

The two-staged phenomenon in ASM strips after length oscillation resembles the sequence of events during DI-induced bronchoprotection in normal subjects. When DIs are immediately followed by administration of a stimulant, airway luminal narrowing is less than it would be without a DI [64] and the airway resistance is reduced [65]. DI protects the airways from excessive bronchospasm. This is similar to the initial reduced contraction observed in ASM when a length oscillation is applied. However, bronchoprotection by DI is temporary. The stimulant-induced bronchospasm gradually returns to what it would be without a DI. This recovery process is paralleled by the *in vitro* finding that the active force of ASM gradually returns to the same level as prior to length oscillation. These similarities between the bronchoprotection of DI *in vivo* and ASM adaptation *in vitro* suggest that the dynamics of ASM length tension behavior and pathologic alterations in this behavior have the potential to play an important role in airway hyperresponsiveness.

The first evidence for a relationship between length adaptation in ASM and AHR was obtained in guinea pig model of maturation. The recovery of active forcein adult guinea pig ASM is gradual, complete, and follows a time course similar to that observed in the ASM from adults of other species. On the other hand, when length oscillation was applied to ASM obtained from airways of 1-wk old guinea pigs, the subsequent active force increased to about 110%$F_{\max}$ ($F_{\max}$: the stable maximal active force generated before mechanical oscillation) and was maintained throughout the adaptation process [66]. This increase of force after the initial reduction was termed force potentiation. These data suggest there is a lack of ASM adaptation in response to mechanical perturbations in immature ASM and is consistent with the clinical observation that airway responsiveness is greater in infants and that DI is ineffective in attenuating airway narrowing in infants as it is in asthmatics [67].

More recently Raqeeb et al. [68] studied ASM *in vitro* using dynamic scenarios which more closely resemble *in vivo* airway mechanics where ASM is constantly subjected to low level length oscillations due to tidal breathing interspersed with occasional DI. In their study design they tested the effect of "tidal breathing" with or without "DI" on force development as well as length oscillation in between stimulations during force recovery. They found that adaptation is interrupted by length oscillations, which suggests that in healthy normal lung where ASM is constantly stretched by breathing motions the force could not reach its maximal level, that is, the second stage of adaptation could not be completed. This would be beneficial to maintain airway patency.

Most recently, Chin et al. [37] directly compared the effect of length oscillation on tracheal ASM strips from nonasthmatic and asthmatic subjects. Immediately after length oscillation ASM from asthmatics showed less force reduction (~half of that in non-asthmatic ASM) and during subsequent recovery the ASM from asthmatics recovered more rapidly and completely. These results suggest that there is a fundamental difference in ASM response to strain: a reduced response in asthmatics to length oscillations; the difference is intrinsic and not because the strain is reduced by stiffer airways. A reduced initial force reduction is consistent with loss of bronchoprotection in asthmatics.

3.2. Subacute/Chronic Length Changes. The effects of subacute and chronic (hours to days) length changes on contractile and structural features have been examined in various skeletal muscle preparations. In the diaphragm, as an example, chronic shortening is clinically relevant in emphysema because the hyperinflation caused by emphysema results in persistent shortening of the muscle. When emphysema is induced in experimental animals by lung elastolysis, the muscle recovers its ability to generate force at short lengths because sarcomeres are subtracted in series over a period of days to weeks [69, 70]. Addition of sarcomeres in series occurs during chronic muscle lengthening [71].

Structural remodeling occurs in asthmatic airways over a long time period and includes increased mucus secretion, excessive deposition of extracellular matrix, thickening of the airway wall, smooth muscle cell hypertrophy/hyperplasia and angiogenesis. These structural alterations could influence airway function by causing ASM adaptation to short lengths. Smooth muscle adaptation to prolonged length changes is much faster than skeletal muscle. It was observed that when ASM strips are passively lengthened or shortened *in vitro* over a period of 24 hr, length-tension (L-T) curves shifted compared to the control curve, allowing maintenance of maximal isometric force at the new length [72]. The result was that smooth muscle adapted to short length was now able to generate the same maximal force as at longer length. Compounding this effect there was a shift of the passive length tension curve to the left, indicating stiffening of the smooth muscle and making reversal of the shortened state more difficult. Naghshin et al. [73] showed that adaptation to passive shortening is reversible after 3 days but not after 7 days. These results suggest that ASM adaptation to shortening can not only occur quickly but also if the shortening conditions persist, more permanent changes can occur.

Despite the general concordance between *in vitro* and *in vivo* studies and modeling some recent work suggests that simple mechanical explanations for the effects of DI may be simplistic. Transmural pressure changes comparable to those produced by tidal breathing do not affect the response of airway segments to a contractile agonist and with amplitudes greater than 10 cm H_2O the airways only respond with a transient dilation [74–77]. Noble and colleagues [75] using isolated airway segments also demonstrated that the capacity of simulated deep inspirations to reduce bronchoconstriction is markedly restricted by stiffening of the airway wall in response to contractile stimulation. Furthermore, the transient airway dilation observed in airway segments is smaller, compared to the relatively larger effect seen in intact airways in vivo [78, 79].

Additionally some *in vivo* studies are not completely concordant with simple mechanical explanations. Although prior DI has reproducibly been shown to differentially modify the methacholine-induced decline in FEV_1 in asthmatics

and nonasthmatics, other studies have shown that there is no differential effect on the changes in FEV_1/FVC ratio [80], partial expiratory flow [81] or airway resistance assessed using the forced oscillation technique (FOT) [82]. One interpretation of this discrepancy is that prior DI may not alter the initial airway narrowing produced by a constrictor but instead make the ASM more responsive to subsequent strain during the DI required to perform an FEV_1 maneuver. This potential mechanism is supported by a recent study of the effect of DI in mice [83].

Together, these data support the idea that the reduced airway response following deep inspiration is likely more complicated than a simple stretch of ASM. One plausible explanation for the ASM response to length oscillation is a corresponding reduction in myosin filament density which has been demonstrated in swine ASM [84]. A similar change in ASM ultrastructure following a DI may explain its bronchoprotective effect. Alterations in myosin filament density in the ASM of asthmatic subjects may make it less prone to disruption following strain, although this has yet to be demonstrated in humans.

Another potential mechanism which could contribute to AHR which involves ASM is the effect of tone on smooth muscle contractility. As mentioned earlier this is an old idea [25] but has received recent attention due to the studies of Bossé and associates [85–88] who used sheep trachealis to model the effect of basal tone on airway smooth muscle's ability to contract [85]. They have shown that tone induced with cholinergic agonists, prostanoids or histamine results in a more than additive effect on subsequent force production in response to electric field stimulation. They have termed this phenomenon force adaptation and have modeled the increased airway narrowing that could come about because of force adaptation [87]. For example, they calculated that force adaptation occurring in an airway of the 9th generation could increase airway narrowing by 48% and airway resistance by 274%. Force adaption is a very plausible contributor to exaggerated airway narrowing in asthmatics given the abundance of inflammatory mediators and spasmogens that the ASM of asthmatics is exposed to. In addition the loss of this tone when the ASM is examined *in vitro* may be one explanation for the failure of excised asthmatic ASM to show altered contractile function in most studies. A recent study by Pascoe et. al. has tested whether force adaptation could influence ASM function in a setting that is more in line with the *in vivo* environment [86]. In this experiment, ASM strips were subjected to force oscillations that mimicked the forces experienced by the ASM *in vivo* during tidal breathing maneuvers with or without deep inspiration. It was shown that even with force oscillations that mimicked *in vivo* breathing patterns, force adaptation still occurred to the same level as in static conditions. This finding opens the door for future *in vivo* work to explore the role of force adaptation in AHR. It is unlikely that force adaptation is the sole cause of AHR but instead is one of a number of components that leads to AHR in asthmatic subjects.

In summary, there is a long history of investigation of the role of ASM in airway responsiveness. Despite this extensive research it remains unclear whether a fundamental change in ASM phenotype is the root cause of hyperresponsiveness [88]. Thus, this series of paper in the Journal of Allergy is pertinent and timely. Since airway, hyperresponsiveness is such a fundamental and clinically relevant characteristic of asthmatic airways it is incumbent on us to definitively incriminate or exonerate ASM.

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Review Article

Airway Smooth Muscle Dynamics and Hyperresponsiveness: In and outside the Clinic

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The primary functional abnormality in asthma is airway hyperresponsiveness (AHR)—excessive airway narrowing to bronchoconstrictor stimuli. Our understanding of the underlying mechanism(s) producing AHR is incomplete. While structure-function relationships have been evoked to explain AHR (e.g., increased airway smooth muscle (ASM) mass in asthma) more recently there has been a focus on how the dynamic mechanical environment of the lung impacts airway responsiveness in health and disease. The effects of breathing movements such as deep inspiration reveal innate protective mechanisms in healthy individuals that are likely mediated by dynamic ASM stretch but which may be impaired in asthmatic patients and thereby facilitate AHR. This perspective considers the evidence for and against a role of dynamic ASM stretch in limiting the capacity of airways to narrow excessively. We propose that lung function measured after bronchial provocation in the laboratory and changes in lung function perceived by the patient in everyday life may be quite different in their dependence on dynamic ASM stretch.

1. Introduction

Excessive and variable airway narrowing is the primary functional impairment observed in patients diagnosed with asthma—usually on a basis of variable wheeze, shortness of breath, cough, and chest tightness that responds well to bronchodilators. In the laboratory this is associated with apparent increased sensitivity (left-ward shift in the dose-response curve) and maximal response to inhaled bronchoconstricting agents [1]. These functional abnormalities are collectively referred to as airway hyperresponsiveness (AHR). In patients with asthma, AHR predicts the susceptibility for an increased rate of decline in lung function [2], increased risk of exacerbations and increased requirements for inhaled corticosteroids [3, 4]. Identifying the mechanism(s) producing AHR in asthma has been a priority research focus over many decades, but our understanding of the pathophysiology of asthma remains incomplete. Explanations for AHR which focussed on the "static" structure-function models of excessive airway narrowing [5, 6] have more recently incorporated the integrated dynamic properties of airway smooth muscle (ASM) [7, 8]. This perspective considers the evidence for and against a role of dynamic ASM stretch in limiting the capacity of airways to narrow excessively, failure of which is proposed as a cause of AHR [9]. Other mechanisms relating dynamic ASM stretch to altered airway calibre include neural, hormonal, and paracrine pathways. These have been summarised previously [10] and will not be discussed in this paper.

2. *In Vivo* Response to Deep Inspiration: Establishing the Hypothesis

The importance of lung volume to airway responsiveness is well recognised: a small increase in volume produces

a substantial reduction in bronchoconstriction [11]. Similarly, studies that alter positive end expiratory pressure (PEEP) also report a strong inhibitory effect of lung volume on bronchoconstriction [12–14]. The role of the dynamic volumes during breathing, as distinct from persist changes in volume occurring with PEEP, is demonstrated *in vivo* by observing how a deep inspiration (DI) transiently stretches the airway wall. Skloot et al. [15] showed that when healthy subjects avoid taking DIs during bronchial challenge, the resulting dose-response curves resembles those of asthmatic patients. That is, the exclusion of DIs from the normal breathing rhythm was seen to increase bronchoconstriction. These findings, combined with observations that the respiratory response to DI is reduced or absent in asthmatic subjects [16–18], and in some patients DI even augments bronchoconstriction [19], suggest that dynamic stretch is an important determinant of airway responsiveness and an abnormality in this protective mechanism could facilitate AHR. However an impaired response to DI in asthma may not account for all features of AHR such as increased sensitivity. The sensitivity of bronchoconstrictor response is not influenced by the presence of DI during provocation challenge [20, 21].

The benefits of DI in healthy individuals include reversal of existing bronchoconstriction (bronchodilation) [20, 22, 23] and attenuation of bronchoconstriction induced following DI (bronchoprotection) [24–26], both of which are reduced in asthmatic individuals [20, 25–27]. The underlying mechanisms of bronchodilation and bronchoprotection may or may not be distinct [24]. The apparent bronchoprotective effects of DI, undertaken prior to bronchial challenge, could involve an enhanced bronchodilatory response since the degree of constriction is measured by the FEV_1 which itself is preceded by a DI and likely to produce bronchodilation [27–29]. Compared with DI, the separate effects of tidal breathing are more difficult to assess, but have been explored in mechanically ventilated animals and are also effective in limiting bronchoconstriction [30–32].

3. Evidence from Isolated ASM

In isolated tracheal ASM strips, the effects of dynamic breathing movements have been simulated by length oscillations prior to or during activation of the ASM, typically by exogenous muscarinic agents or parasympathetic nerve stimulation [8, 33–38]. Length oscillation during ASM activation resulted in a marked decrease in ASM force production [8, 33, 38] and shortening [34], in proportion to the amplitude of length oscillation. Importantly, it is proposed [8] that large changes in ASM force will occur to length oscillation accompanying tidal breathing (estimated from lung volumes and assuming isotropic expansion). Cellular mechanisms include cross-bridge detachment due to lengthening, the so-called "perturbed equilibrium hypothesis" [34, 39].

Length oscillation of ASM prior to activation is also effective in modulating ASM force and this is similarly dependent on the amplitude of length change [35–37]. Plasticity of ASM force-length properties (length adaptation) has been evoked to explain the effect of length change on the relaxed cell via remodelling of the contractile apparatus [40–42]. Length adaptation or plasticity at least theoretically explains both the bronchodilatory and bronchoprotective effects of DI [43, 44].

4. Evidence from Bronchial Segments

Whole bronchial segments that retain the normal architecture of ASM and connections with other mural components have been used to study the effect of dynamic stretch on ASM contraction. Gunst et al. [45] applied fixed volume oscillations to canine airway segments and examined the effects of bronchoconstriction to acetylcholine. They showed a pronounced reduction in the contractile response (narrowing and pressure generation) during volume oscillation, findings qualitatively similar to those in isolated ASM *in vitro* [8, 33, 38] and in mechanical ventilated animals *in vivo* [30–32]. Subsequently, numerous other studies using porcine airway segments confirmed that volume oscillations suppress bronchoconstriction [46–48]. However, what is clear is that pressures accompanying volume oscillation in airway segments become very large during contractile activation, a function of ASM stiffening [49].

We found that although baseline transmural pressures and volumes were chosen to simulate tidal breathing in the relaxed airway (i.e., $\Delta P = 5\,\mathrm{cmH_2O}$), the pressure swings associated with fixed volume oscillation during ASM stimulation increased ~four fold (Figure 1). When volume oscillations that produced more physiological pressure swings were used during ASM activation the effect of oscillation was greatly attenuated [48]. These observations lead us to conclude that the effects of dynamic stretch are limited by wall stiffness and that tidal oscillations are unlikely to significantly impact airway responsiveness. This conclusion was supported by LaPrad et al. [50] who applied fixed pressure oscillations on bovine airway segments and measured the effect on airway narrowing measured by ultrasound imaging. Under fixed pressure conditions airway narrowing was unaffected by tidal oscillations, casting doubt on the role of tidal oscillations in determining airway responsiveness [51].

Bronchial segment studies have also examined the effect of short-term inflations simulating DI, typically defined as inflation to $30\,\mathrm{cmH_2O}$ which corresponds to transpulmonary pressure at the plateau of the lung pressure volume relationship [52]. In porcine airway segments DI produces potent, transient bronchodilation, largely dissipating within ~1 min [53, 54]. The magnitude and time-course of the bronchodilatory response in airway segments are consistent with bronchodilation to DI observed *in vivo* [22] suggesting that the airway wall response to dynamic stretch mediates this effect. Bronchodilatory responses to DI in whole airways are inversely proportional to airway wall stiffness and proportional to the magnitude of ASM stretch [48].

The level of ASM activation induced *in vitro* clearly impacts on the response to dynamic respiratory manoeuvres. Notably, bronchodilatory responses to DI in airway segments are observed under submaximal narrowing conditions (~30–40% decrease in lumen area) [53, 54] which

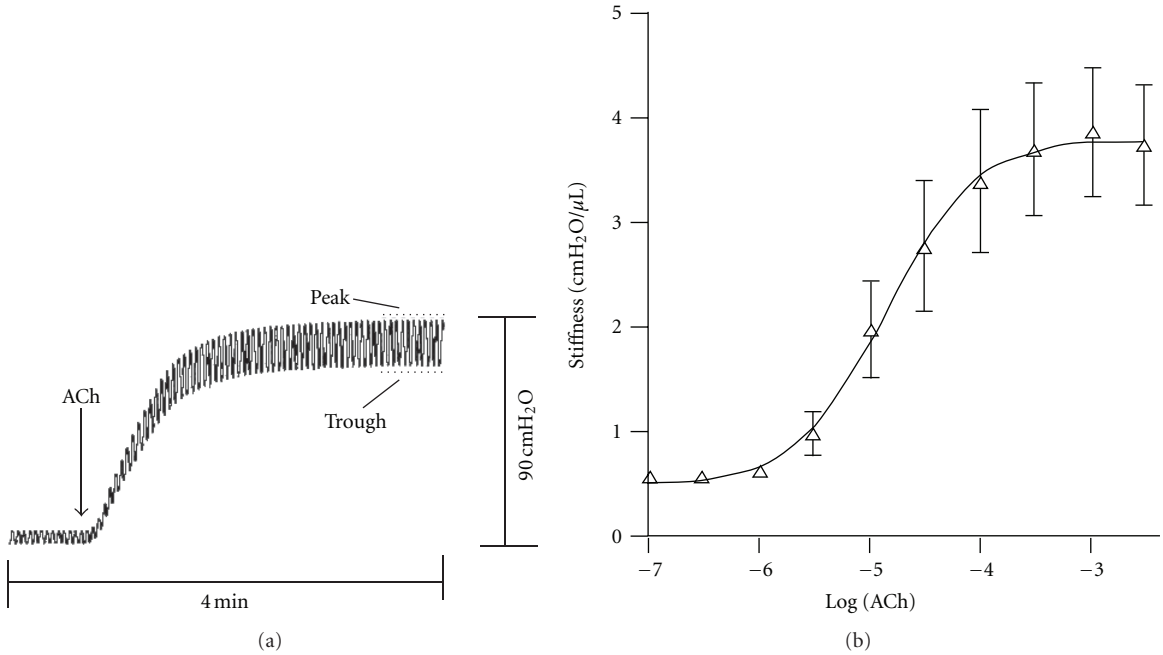

Figure 1: From [48]. (a) Lumen pressure fluctuations in isolated bronchial segments (porcine) during tidal volume oscillation before and after a maximal dose of acetylcholine (ACh). Tidal volume oscillations produced a trough-to-peak pressure cycle from 5 to 10 cmH_2O in relaxed airways. Contraction to ACh is seen by the elevation in trough pressure in a closed system. The increase in the amplitude of pressure cycles indicates stiffening of the airway wall to ACh. (b) Sigmoidal dose-response behaviour of ACh-induced increase in airway stiffness. Values are means ± SE ($n = 5$).

should still be sufficient to produce large reductions in flow (~50–60% assuming homogenous constriction and laminar flow). However bronchodilatory responses to DI become diminished with increasing levels of ASM activation [48], and conversely, fixed pressure tidal oscillations can be effective under levels of activation at the bottom of the *in vitro* dose-response curve [53]. A question thus arises whether examining the response to dynamic mechanical stretch at maximal or near maximal levels *in vitro* is more relevant to disease, that is, asthma.

The animal models used in studies utilising airway segments and muscle strips introduce the question of possible species differences in the role of dynamic ASM strain [55]. There are some differences between species that could impact the response to tidal or DI breathing, for example the porcine airway has a more abundant cartilaginous wall than the human airway which increases stiffness [56], while the bovine airway exhibits a myogenic response to simulated DI [50, 57] seemingly more in line with the bronchoconstrictor response after DI observed in some asthmatic patients [19]. Translational studies using human tissue are therefore necessary to confirm or extend findings in animal models. As discussed below, broadly speaking there is good agreement between studies utilising human tissue with those working with animal models.

5. Human Tissue

To our knowledge four studies have reported the responses of human ASM to dynamic stretch. Tracheal ASM from nonasthmatic nontransplantable human lungs [58] showed attenuated force production following length oscillation, confirming findings from animal models. The same group also reported that in subjects with asthma the protective response to length oscillation was partially impaired [59]. This suggests that the reduced response to DI in asthmatic subjects may result from an impaired response of the ASM to mechanical stretch.

We examined the effects of simulated breathing manoeuvres in human bronchial segments [60] using tidal oscillations and DIs that mimicked the fixed pressure swing protocols described above [50, 53, 54]. Airway narrowing in tidally oscillated airways was reversed immediately after DI (Figure 2), followed by reconstriction over the course of 1 min. While the study did not examine the independent effects of tidal oscillation, the level of airway narrowing before the initiation of DI was similar to that under static conditions, arguing against an effect of tidal oscillation on airway narrowing.

A human lung slice model has been used recently to examine the effect of tidal and deep breathing on airway narrowing [61]. A constant stress perturbation was applied to the lung slice, thereby imparting strain to the airway wall. The major results of the study confirm many of the previous findings from both animal and human tissue. Airway narrowing was reversed by deep "breathing" but not smaller "breaths." In particular, tidal oscillations were ineffective. The effectiveness of breathing to antagonise airway narrowing increased with the level of wall stretch and decreased with the greater levels of contractile activation and wall stiffening.

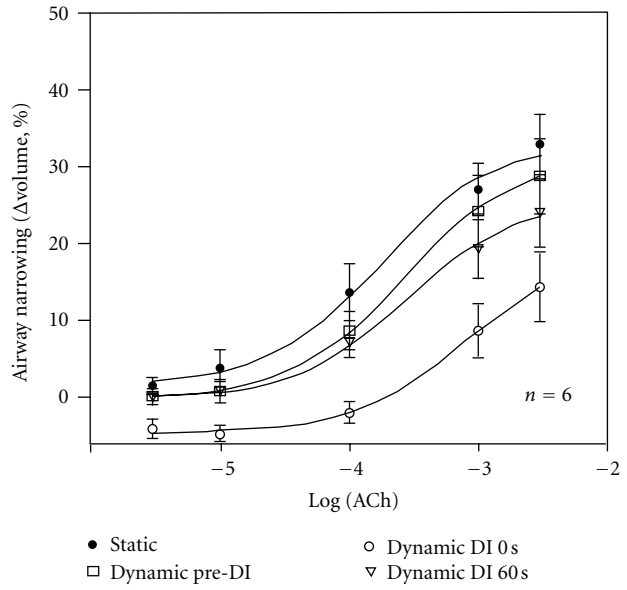

Figure 2: From [60]. Airway narrowing (Δvolume, %) to acetylcholine (ACh) in human bronchial segments. ACh dose-response curves were constructed from measurements of airway narrowing under static conditions (Static, 5 cmH_2O) and during fixed transmural pressure cycles simulating tidal (5 to 10 cmH_2O at 0.25 Hz) and deep inspiration (DI, 5 to 30 cmH_2O). The dynamic pre-DI curve represents airway narrowing before the onset of DI; dynamic DI 0 s, the airway narrowing measured immediately after DI; dynamic DI 60 s, the airway narrowing measured 1 min after DI. DI produced an immediate reduction in maximal airway narrowing ($P < 0.001$) but not sensitivity. The effects of DI were largely ablated after 1 min. Airway narrowing under static conditions was not different to that prior to DI, suggesting that tidal oscillations alone did not regulate airway narrowing. Values are means ± SE ($n = 6$).

6. ASM Dynamics and AHR in the Lung Function Laboratory

Many of the effects of DI are reasonably explained by the responses to dynamic stretch observed in isolated ASM and airway tissue *in vitro* and these will impact on measurements of airway responsiveness in the clinic. That is, since the traditional measure of airway responses to bronchoconstrictor agents is the FEV_1, the DI which precedes the forced expiratory manoeuvre will produce bronchodilation and a differential response to DI between healthy and asthmatic subjects will result in a divergence of the dose-response curves [20]. Certainly, bronchodilator responses to DI are transient [17, 22], but in the context of a conventional bronchial challenge any bronchodilation, no matter how short-lived, will influence the FEV_1 parameter. Extrapolating from the behaviour of individual airways [60], the magnitude of this effect approaches a halving in maximal response (Figure 2).

The mechanism producing AHR is however more than just an abnormal response to DI. Abnormal bronchodilatory responses to DI cannot explain AHR when constrictor responses are measured without the need for a DI (as required for FEV_1) such as the forced oscillation technique [62]. As discussed, the effects of DI also do not explain any increased sensitivity of response in asthma since the position of the dose-response curve is not altered by the presence of DI during bronchial challenge [20, 21]. Some authors argue that the effect of DI in regulating the maximal response to bronchoconstrictor challenge in the clinical laboratory may "*artificially enhance the differences in responsiveness between healthy and asthmatic subjects*" [51]. Outside the boundaries set within the lung function laboratory, and putting FEV_1 aside which provides just a snapshot of airway function, the dependence of lung function on DI as perceived by the patient may be quite different.

7. ASM Dynamics and AHR outside the Lung Function Laboratory

Debate remains regarding whether airway responsiveness (the capacity for airways to narrow and restrict airflow) is suppressed by tidal oscillations and regular deep breaths, occurring in the form of spontaneous sighs at a rate of one in every six minutes [63]. The bronchodilatory responses to DI will of course have some effect but whether such transient bronchodilator events, which as discussed are influential in the measurement of the clinically derived FEV_1 parameter, are frequent enough to be of major consequence to a patient in everyday life is uncertain. With respect to tidal oscillation, the evidence is mounting that under conditions where pressure fluctuations across the airway wall are constant (the scenario which is expected to occur with tidal breathing *in vivo*) these perturbations will have little to no effect on airway narrowing [48, 50, 60, 61].

The above leads to a conclusion that in the context of set limits of stiffness and strain the dynamic environment plays an important role in the measurement of airway responsiveness, as performed in the laboratory. But, given the kinetics of the dynamic response, perhaps it is unlikely to play a role in the day-to-day symptoms of the patient, that is, feelings of wheeze or chest tightness experienced by asthmatic individuals.

We need to try and resolve the apparent discrepancies between findings *in vivo* and *in vitro*. Tidal oscillations in mechanically ventilated animals *in vivo* demonstrate physiologically meaningful effects on airway narrowing [30–32] which is inconsistent with studies in isolated airways and lung slices *in vitro* [48, 50, 60, 61]. Interestingly, it was only since mechanical "limits" (i.e., pressure) were superimposed on our biological models that the effects of dynamic stretch appeared less effective [48]. Do pressure oscillations remain fixed during contractile activation *in vivo*? In studies on mechanically ventilated animals, it is tidal *volume* rather than pressure that is held fixed and this may account for the greater potency of tidal oscillations in this scenario. As the impedance of the system is increased with bronchoconstrictor challenge, respiratory pressures would be expected to increase for a constant volume change as observed in mechanically ventilated dogs [31]. Alternative explanations have also been proposed including an elevation in mean airway pressure during mechanical ventilation [50], however, this possibility has been empirically tested and

only partially explains beneficial responses to tidal volume oscillations [31].

If we then consider what happens when a patient undergoes bronchoconstriction, in order to maintain adequate minute ventilation respiratory pressures may also increase to overcome the greater system impedance, although this will be influenced by the magnitude of the bronchoconstrictor response. Perhaps the true physiological simulation is one that exists somewhere between the fixed volume and pressure protocols previously described. The true biological effect of tidal oscillation then exists somewhere between these limits and may be greater than what has been suggested in recent studies [48, 50, 60, 61]. Indeed regular deep breathing is effective in reversing induced bronchoconstriction [64].

A final consideration is how bronchoprotective effects of DI influence bronchoconstriction *in vivo*. Unlike isolated ASM which exhibits a bronchoprotective-like effect whereby prior mechanical stretch reduces ASM force [35–37], bronchoprotection is not observed in midsized whole airways *in vitro* [60, 65]. The response of the whole airway is consistent with global *in vivo* measures of airflow and resistance that reveal no protective effect of prior DI on airway narrowing [27, 28, 66, 67]. The bronchoprotective effects of DI instead reduce the tendency towards airway closure, possibly by reducing airway surface tension [66]. The mechanism of DI-induced bronchoprotection may therefore involve more than an effect of mechanical stretch on the ASM.

8. Beyond ASM Dynamics

The role of ASM dynamics in the development of AHR should not be considered in isolation from other likely mechanism(s) including the effect of a thickened ASM layer in asthma [68]. The most intuitive explanation for an increase in maximal airway narrowing is enhanced ASM force due to greater ASM mass. This possibility is supported by mathematical simulations [5] but still lacks confirmatory biological data. The importance of ASM mass to AHR was well demonstrated using a murine gene knockout model of early growth response-1 which following stimulation with transforming growth factor alpha has pronounced ASM thickening and a severe form of AHR (compared with other models) [69]. The ASM growth was attributed solely to ASM hyperplasia which is the predominant pathology seen in severe asthma [70].

Neither increased ASM mass nor altered ASM dynamics account for changes in airway sensitivity. On reflection this is not surprising given the fact that mechanisms controlling sensitivity and maximal response (of the ASM and intact airways) differ [6]. The role of the epithelial mechanical barrier in limiting sensitivity to bronchoconstrictor stimuli was demonstrated decades ago by use of whole bronchial airway models *in vitro* [71, 72], similar to those described elsewhere in this paper. In intact airways the accessibility of ASM to agents applied to the airway lumen provides one of the strongest regulators of sensitivity. The original studies have been revisited recently in a mouse model [73].

9. Concluding Statements

The evidence from studies examining isolated ASM and whole airway behaviour *in vitro* suggests that dynamic ASM stretch is one determinant of airway responsiveness. The magnitude of this effect is dependent on the limits of these biological models including the magnitude of airway stretch, stress and ASM activation. It is unclear whether the effects of dynamic ASM stretch observed in the context of lung function measurements also influence the clinical symptoms of asthma. However, dynamic ASM stretch is unlikely to be the sole determinant of airway responsiveness and any impairment of this regulatory mechanism will interact with other pathological changes to produce AHR.

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Review Article

Can We Find Better Bronchodilators to Relieve Asthma Symptoms?

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Bronchodilators are the first line therapy during acute asthmatic exacerbations to reverse airway obstruction primarily by relaxing airway smooth muscle. Only three categories of bronchodilators exist in clinical practice: β-adrenergic agonists, anticholinergics, and methylxanthines. Each of these categories have specific drugs dating back to the early 20th century, raising the question of whether or not we can find better bronchodilators. While caffeine, theophylline, atropine, and epinephrine were the first generations of therapeutics in each of these drug classes, there is no question that improvements have been made in the bronchodilators in each of these classes. In the following editorial, we will briefly describe new classes of potential bronchodilators including: novel PDE inhibitors, natural phytotherapeutics, bitter taste receptor ligands, and chloride channel modulators, which have the potential to be used alone or in combination with existing bronchodilators to reverse acute airway obstruction in the future.

1. Novel PDE Inhibitors

Caffeine and theophylline are two methylxanthines that have been used to treat asthma, although their exact mechanisms of action are still unknown [1, 2]. The bronchodilating property of theophylline is largely attributed to inhibition of phosphodiesterase (PDE) activity or the release of catecholamines, thereby increasing cAMP in airway smooth muscle [3]. In use since the 1930s, lack of specificity, a narrow therapeutic window, and negative side effects, plus the development of inhaled glucocorticoids, and short- and long-acting β_2-agonists have decreased the clinical utility of theophylline [1, 4, 5]. However, the identification of airway-specific PDE4 subtypes and subsequent development of PDE4-selective inhibitors have resurrected this avenue as novel bronchodilators.

The newest PDE4 inhibitor approved for use in respiratory disease is roflumilast (Daxas) [6, 7]. In patients with COPD, roflumilast has shown significant improvement of FEV1 when taken in conjunction with long-acting β_2-agonists or muscarinic antagonists [8]. In preclinical studies, administration of selective PDE4 inhibitors prevented bronchial hyperresponsiveness (BHR) in allergic mice, an *in vivo* model for asthma [9, 10]. Although no PDE4-selective inhibitor is currently approved for the treatment of asthma in the United States, similar studies showed that administration of PDE4 inhibitors attenuated BHR in patients with allergic asthma [11–13]. With improved specificity for PDE4 subtypes, PDE4-selective inhibitors (roflumilast, CDP840, MK-0359), have decreased side effects compared to theophylline although the therapeutic window is still narrow and still produces systemic effects due to oral administration. Development of inhaled PDE4-selective inhibitors has shown potential in reducing early- and late-phase asthmatic responses in mild allergic asthmatics [14]. Whether or not these improvements in FEV1 are due to bronchodilation of airway smooth muscle or anti-inflammatory effects are yet to be determined; however, inhalation will likely reduce plasma levels of drug and decrease side effects associated with oral delivery. Further work on developing PDE4 subtype-specific inhibitors (A–D) or combining various PDE isoform inhibitors (i.e., PDE1, 3, 7 with PDE4

inhibitors) [5] may increase the efficacy of targeting this signaling pathway in treating asthma, providing a new application for a longstanding bronchodilator.

2. Natural Phytotherapeutics

Of note, one PDE4-selective inhibitor, quercetin, is a naturally occurring flavonol found in fruits, vegetables, and tea leaves. Retrospective studies have shown increasing numbers of asthmatics self-treat their symptoms with herbal remedies [15, 16]. In many cases, the exact mechanisms of action of these natural botanicals are unknown; however recent work has focused on identifying the active constituents of herbal remedies and elucidating the signaling pathways involved in acute bronchodilation. Given the advances in PDE inhibition and the natural origin of many methylxanthines, many of these natural phytotherapies may possess PDE inhibitory action.

Recently, natural plant products have received accolades for the treatment of cough, respiratory infection, and bronchospasm [17]. It is estimated that 10%–42% of asthmatics use herbal therapies to self-treat their asthma symptoms [16, 18]; however the efficacy and safety of most herbal therapies have not been scientifically evaluated [19]. The exact mechanism of action of most of these agents is unclear but may involve direct effects on airway smooth muscle, airway epithelium, airway nerves, inflammatory cytokines, and immune cells. Moreover, the formulations of these herbal compounds are made up of many individual bioactive compounds. As such, it is important to define both the positive and potential negative impacts of these individual compounds on the airway as well as explore the interaction of herbal therapies with existing asthma therapies (corticosteroids and β-agonists).

Extensive preclinical, animal, and human studies have demonstrated that antiasthma simplified herbal medicine intervention (ASHMI), an extract of 3 plants *Ganoderma lucidum* (Ling-Zhi), *Sophora flavescens* (Ku-Shen) and *Glycyrrhiza uralensis* (Gan-Cao), reduces lung inflammation, airway remodeling, and airway smooth muscle hyperresponsiveness [20–22]. A blinded randomized trial in 91 subjects with moderate to severe allergic asthma demonstrated that 4 weeks of oral ASHMI were nearly equivalent to oral prednisone in the improvement in FEV1, peak flows, serum IgE levels, and eosinophilia [23]. The safety and tolerance of oral ASHMI were confirmed in a dose escalation study [21]. These clinical studies were followed by a series of preclinical studies that sought to identify the mechanism(s) involved in the improvement of symptom and inflammatory profiles. Both chronic and acute beneficial effects of ASHMI were demonstrated on mouse lung inflammation and responsiveness. Six weeks of oral administration of ASHMI reduced inflammation and *in vivo* responses to acetylcholine [20, 22, 24]. Acute treatment of isolated tracheal rings with ASHMI from naïve or ovalbumin sensitized mice demonstrated reduced acetylcholine-induced contractions in *ex vivo* organ bath experiments [22]. A possible mechanism for these acute effects was elucidated in human airway smooth muscle cells that liberated prostaglandins in response to ASHMI [22], which could mediate relaxation through activation of G_s-coupled EP2 or EP4 receptors [25]. Current research is focused on identifying the specific purified chemical constituents of ASHMI that mediate these chronic anti-inflammatory effects and acute airway smooth muscle relaxant effects.

Although PGE_2 relaxes airway smooth muscle in many species and benefits of inhaled PGE_2 have been shown in asthmatics, a specific agonist for the EP2 receptor failed to show benefit in human trials [26]. However, newer studies suggest that targeting the EP4 receptor in human airway smooth muscle may be an alternative therapeutic target in patients with asthma [27].

3. Bitter Tastants

Another potential therapeutic target in the treatment of bronchoconstrictive disease involves the bitter taste receptor family (TAS2R). Recently, both qRT-PCR analysis and immunofluorescence microscopy of human airway smooth muscle (ASM) cells revealed robust expression of several members of this G-protein-coupled receptor family (TASR-10, -14, and -31) and showed increases in intracellular calcium ($[Ca^{2+}]_i$) in response to subsequent exposure to bitter tastants, the agonists to these receptor subtypes [28]. Despite increasing ASM $[Ca^{2+}]_i$ via the same pathway ($G\beta\gamma \rightarrow PLC\beta \rightarrow IP_3R$) shared by the classical contractile agonist acetylcholine, this group paradoxically found activation of TAS2R in ASM leads to a profound degree of ASM bronchodilation in both isolated ASM preparations as well as *in vivo* models of induced airway responsiveness. Interestingly, the magnitude of bronchodilation achieved by high-dose TAS2R agonists in many of these studies rivaled maximal β-agonist treatments and mechanistically was found to be cAMP- and PKC-independent. This group has recently extended this observation to show that in relevant models of β_2-adrenoceptor desensitization, chloroquine-mediated TAS2R activation in ASM retains its pro-bronchodilatory effects, a finding of considerable clinical relevance given the well-described concern of β-agonist tachyphylaxis that occurs with repetitive β-agonism [29]. Yet, it should be noted that TAS2R activation in ASM can lead to desensitization via a GRK-mediated, β-arrestin pathway, which may limit its therapeutic usefulness as it is seen currently with β-adrenoceptor agonists [30].

Mechanistically, TAS2R activation in ASM is thought to achieve relaxation via a localized $[Ca^{2+}]$-dependent activation of the large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) leading to membrane hyperpolarization. While other investigators have challenged the notion that bitter tastant-mediated ASM relaxation is BK_{Ca}-dependent [31], the evidence in human ASM suggests at least a partial role of the BK_{Ca} channel in what is likely a novel, multimodal mechanism leading to ASM relaxation [32]. The possibility of TAS2R activation in ASM (in the context of localized calcium release) leading to non-BK_{Ca}-mediated ASM relaxation via yet undescribed pathways is another exciting prospect behind

Table 1: Summary of benefits and limitations of novel bronchodilators.

Drug class	Benefits	Limitations
β-agonists	Rapid airway relaxation Selective for β_2-AR; decreased systemic effects	Receptor desensitization Receptor downregulation Refractory bronchoconstriction Asthma-related death
PDE inhibitors	Increase cAMP generated endogenously Enhance β_2-AR effects Selectivity for subtypes specific to lung	Oral delivery Complex dosing and metabolism Systemic side effects Potential adenylyl cyclase and/or β_2-AR desensitization
Phytotherapeutics	Airway relaxation Acute and chronic effects Reduces inflammation and remodeling Increased patient compliance	Mechanisms of action are not clearly defined Potential interaction with other drugs Difficulty standardizing source and dosing
Bitter tastants	Novel target—may augment traditional therapies due to cAMP-independence	Mechanisms of action are not clearly defined
Chloride channel modulators	Novel target—may augment traditional therapies May address neuronal components of airway tone	Mechanisms of action are not clearly defined Method of delivery Interaction with airway epithelium (mucous production) Systemic side effects

this work that may uncover other potent targets to facilitate relaxation not susceptible to GPCR tachyphylaxis.

4. Chloride Channel Modulators

Chloride channels are expressed on airway smooth muscle and have been shown to effect airway smooth muscle force [33] and cell length [34]. In 2005, Hirota et al., described attenuation of acetylcholine-induced contractions in ASM subsequent to calcium-activated chloride channel antagonism [33]. Additionally, activation of the ligand-gated chloride channel, $GABA_A$, relaxed airway smooth muscle precontracted with the tachykinin, substance P [35]. In 2011, another ligand-gated chloride channel, the glycine receptor, was shown to relax airway smooth muscle contracted with a selective neurokinin 2 receptor agonist [36]. These and other studies have led to the understanding that chloride channels may play a significant role in the airway smooth muscle contraction and relaxation mechanisms.

Calcium-activated chloride channels have been described functionally; however, the true molecular identity of calcium-activated chloride channels have only recently been identified as belonging to the ANO or TMEM16 receptor family. The TMEM16 receptors are membrane proteins with 8 transmembrane domains shown to allow chloride flux in the presence of increasing calcium while possessing voltage sensitive activity. TMEM16A mRNA expression has been described in airway smooth muscle [37] and its function in other cell types has been described as contributing to membrane depolarization during calcium increases [38]. It has been hypothesized that acetylcholine- and caffeine-mediated release of calcium from the sarcoplasmic reticulum (SR) stimulates chloride efflux from the cell, leading to depolarization of the plasma membrane. Force studies in *ex vivo* airway smooth muscle preparations examining the effects of chloride channel antagonists, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and niflumic acid (NFA), showed a large attenuation of acetylcholine-induced contraction by NPPB while NFA failed to have an effect. In contrast, caffeine-induced contractions were inhibited by both NFA and NPPB [33]. The differential effects of these chloride channel antagonists may be due to the effects on calcium-activated chloride channels located on the SR versus the plasma membrane. Recently, members of the TMEM family were shown to be expressed on various intracellular compartments and not exclusively on the plasma membrane [39]. A possible mechanism of attenuated force generation in airway smooth muscle by calcium-activated chloride channel antagonism may be inhibition of chloride ion efflux during contractile agonist stimulus.

Ligand-gated chloride channels have been well described in the central nervous system with two families dominating the role as inhibitory inputs, $GABA_A$, and glycine receptor channels. Both $GABA_A$ and glycine receptors are expressed in ASM and possess functional roles in the modulation of airway smooth muscle tone generation [35, 36]. This inhibitory effect on ASM contraction may be attributed to a relative hyperpolarization of the membrane potential after it has surpassed the chloride reversal potential following exposure to a contractile stimulus. This opening of the chloride channels causes an influx of chloride ions leading to a relative membrane hyperpolarization, eliminating, or attenuating the electromechanical component of contraction. Additional studies have described the importance of specific $GABA_A$ receptor subunits. $GABA_A$ channels containing alpha4 or alpha5 subunits can be selectively targeted in airway smooth muscle resulting in effects on membrane potential and airway tone [40]. Pharmacotherapies that are not $GABA_A$ subunit selective, such as the general anesthetic, propofol, have bronchodilatory capabilities [41]. Increased airway smooth muscle specificity will determine the viability

of $GABA_A$-related therapies as bronchorelaxants. $GABA_A$ function in airway smooth muscle has been studied in both rodent and human *ex vivo* models, as well as *in vivo* rodent models [42, 43], producing strong evidence that this channel, once thought to be exclusively neuronally expressed, may have direct effects on airway tone.

In the last ten years, the existence of chloride channels in airway smooth muscle has been confirmed yet our current understanding of their mechanistic and functional roles remains incomplete. Although poorly mechanistically understood [44, 45] manipulation of chloride channels still remains a viable avenue of further research in the discovery of novel bronchodilators. Continued research will uncover the exact mechanisms that dictate the role for chloride channels in the balance of contraction and relaxation in the airway.

While bronchodilators will likely continue to be a mainstay of asthma therapy far into the future, the classical relaxant, β-agonist, is not without limitations. Receptor desensitization, β-agonist insensitivity, β-agonist refractory bronchoconstriction, and even death are all risks associated with prolonged use of traditional β-agonists. As such, it is important to continually investigate new therapeutics for the treatment of asthma; keeping in mind that acute bronchodilation is the first line therapy during an asthmatic episode. Here we have illustrated 4 novel potential therapeutics that show functional bronchodilatory properties in the airway owing to a variety of mechanisms. These novel compounds may augment existing β-agonist relaxant effects as in the case of PDE inhibitors or provide complementary avenues for relaxation when combined with current therapies. Table 1 summarizes beneficial aspects of traditional β-agonists and these novel therapeutics as well as illustrating current limitations to implementing these novel bronchodilators. Interestingly, compounds that transiently elevate $[Ca^{2+}]_i$ such as phytotherapeutics, bitter taste ligands, $GABA_A$ receptor ligands, and chloride channel antagonists subsequently lead to functional relaxation of airways. This is counterintuitive in the face of decades of research closely linking global cellular calcium and smooth muscle contraction thus necessitating a broader understanding of complex calcium dynamics within cellular microdomains. While the mechanism of action of these potential therapeutics is still under investigation, they open the door for assessing new therapeutics and mechanisms leading to bronchodilation.

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Review Article

Integrin and GPCR Crosstalk in the Regulation of ASM Contraction Signaling in Asthma

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Airway hyperresponsiveness (AHR) is one of the cardinal features of asthma. Contraction of airway smooth muscle (ASM) cells that line the airway wall is thought to influence aspects of AHR, resulting in excessive narrowing or occlusion of the airway. ASM contraction is primarily controlled by agonists that bind G protein-coupled receptor (GPCR), which are expressed on ASM. Integrins also play a role in regulating ASM contraction signaling. As therapies for asthma are based on symptom relief, better understanding of the crosstalk between GPCRs and integrins holds good promise for the design of more effective therapies that target the underlying cellular and molecular mechanism that governs AHR. In this paper, we will review current knowledge about integrins and GPCRs in their regulation of ASM contraction signaling and discuss the emerging concept of crosstalk between the two and the implication of this crosstalk on the development of agents that target AHR.

1. Introduction

Airway hyperresponsiveness (AHR) is the exaggerated response to relatively low concentrations of constricting agents (such as methacholine or histamine) or indirectly acting stimuli (such as cold air, respiratory infections or allergens, exercise, or cigarette smoke) that is observed in asthmatic subjects [1]. Contraction of airway smooth muscle (ASM) cells that line the airway wall is thought to influence aspects of AHR, culminating in the generation of force and excessive narrowing or occlusion of the airway [2]. ASM contraction is primarily controlled by agonists that bind G protein-coupled receptors (GPCR), which are expressed on ASM. Studies have shown that the asthmatic airways can be completely occluded even with only 40% contraction of ASM cells following an asthma exacerbation to GPCR agonists, such as histamine, that induce muscle shortening [3, 4]. Therefore, ASM GPCRs are important targets for therapeutic agents in asthma treatment. However, there is increasing evidence to suggest that chronic use of β_2-adrenergic receptor agonists, which act on GPCRs, is associated with worsening of bronchoconstrictor response to airway spasmogen [5], loss of asthma control, and exacerbation of asthma symptoms [6, 7], as well as an increased incidence of asthma-related morbidity and mortality [8]. Moreover, glucocorticoids, which are used as first line therapy for the treatment of inflammation associated with asthma, decrease AHR only if introduced early in disease diagnosis [9, 10]. Even then, there are side effects associated with the use of glucocorticoid when used at high dose and over long periods [10, 11]. Thus, the current treatment for asthma is based on symptom relief only and the ultimate goal of treating asthma is to target the underlying mechanisms, which include AHR.

We and others have shown that integrins may influence signaling events that contribute to AHR [12–14]. However, the mechanism behind this regulation remains to be fully elucidated. Moreover, there is increasing evidence to show that GPCRs interact with integrins to regulate ASM signaling pathways that are important in asthma. The cellular signaling processes include the regulation of cell adhesion, calcium signaling, injury and remodeling, mechanotransduction signaling and synaptic plasticity [15–18]. In this paper, we

will review current knowledge about integrins and GPCRs in their regulation of ASM contraction signaling and discuss the emerging concept of crosstalk between the two and the implication of this crosstalk on the development of agents that target AHR.

2. Integrins and ASM Contraction Signaling

Integrins are heterodimeric transmembrane proteins comprising one α and β chain. The expression of different integrins in ASM, their potential ligands and change in expression in asthma are detailed in Table 1. Integrin activation via ECM protein binding leads to the formation of a complex called focal adhesion, which consists of many structural proteins such as vinculin, talin, α-actinin, and paxillin [19–21]. Integrins can signal through the cell membrane in both directions: inside-out signaling and outside-in signaling. The extracellular binding activity of integrins is regulated from the inside of the cell (inside-out signaling), while the binding of ECM proteins such as laminin elicit signals that are transmitted into the cell (outside-in signaling) [22]. It is through these signaling activation events that integrins regulate cell attachment, survival, proliferation, cell spreading, differentiation, cytoskeleton reorganization, cell shape, cell migration, gene expression, tumorigenicity, intracellular pH, and increase in concentration of cytosolic Ca^{2+} [23].

Activation of integrins by either contractile or mechanical stimuli can result in two signaling events to cause ASM cell contraction. Firstly, integrin activation causes the phosphorylation of focal adhesion kinase (FAK) and association with paxillin, leading to reorganization of the cytoskeleton [24–26]. Secondly, integrin activation will also increase intracellular Ca^{2+} concentration causing the phosphorylation of myosin light chain kinase (MLCK) and activation of myosin ATPase activity, and crossbridge cycling [24–26].

3. GPCR and ASM Contraction Signaling

GPCR spans the cell membrane seven times and transduces extracellular stimuli from the binding of cell surface ligands into intracellular second messengers. These second messengers are known as the heterotrimeric guanine nucleotide-binding protein (G proteins), which consists of G_α, G_β, and G_γ subunits [39]. G proteins bind to the intracellular domain of GPCR and transmit signals that are important in ASM cellular functions. These functions include regulation of ASM proliferation and secretion of cytokines, chemokines, eicosanoids, or growth factors that orchestrate airway inflammation and remodeling [40]. GPCRs are also implicated to play important role in ASM cell contraction. The regulation of ASM tone is mediated by a balance between G_q- and G_s-coupled signaling, with G_q being linked to ASM contraction signaling and G_s being linked to relaxation signaling [40–43]. Agonist binding causes the activation and association of GPCRs with G_q, which promotes GTP binding and dissociation of G_α from $G_{\beta\gamma}$ subunits. The dissociated G_q will then bind to effector phospholipase C, which then hydrolyses phosphoinositol 4,5-bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). The net effect of these events is to increase the levels of intracellular Ca^{2+} as well as to activate cell contractile machinery through Ca^{2+} and protein kinase C- (PKC-) dependent mechanisms [40]. Activated PKC is able to phosphorylate a number of substrates which include calponin [41]. Phosphorylated calponin loses its ability to inhibit actomyosin ATPase, which is required for ASM cell contraction [41].

4. Evidence for Integrin and GPCR Crosstalk

There is emerging interest in crosstalk between integrins and GPCRs (Table 2). For example, muscarinic agonists that bind $G_{12/13}$ protein can induce FAK activation and autophosphorylation in Swiss 3T3 cells, a fibroblast cell line, which is associated with integrin engagement signaling [52]. Arg-Gly-Asp (RGD) is a consensus amino acid sequence found in ECM proteins that is recognized by integrins. It is found that muscarinic-induced FAK activation can be blocked by RGD peptide, suggesting crosstalk between GPCRs and integrins [52]. Similar observations have been observed for other GPCR agonists such as gastrin, endothelin, lysophosphatidic acid (LPA), angiotensin II, and bombesin [23, 53–56]. For example, stimulation of Swiss 3T3 cells with bombesin or endothelin results in FAK and paxillin phosphorylation and accompanied formation of focal adhesion plaques. This study suggests the formation of focal adhesion plaques as a common signal transduction pathway that mediates GPCR and integrin crosstalk. As for endothelial cells, angiotensin II is able to induce FAK and paxillin phosphorylation which results in augmented cell migration necessary for blood vessel repair and wound healing. This suggests a critical role for integrins in the angiogenic effect of angiotensin II via FAK activation. Taken together, the existence of distinct pathways leading to FAK activation suggests the possibility of synergistic interaction between GPCRs and integrin receptors. One of the key signaling events following integrin ligation is the activation of FAK. FAK activation recruits phosphatidylinositol-3-kinase (PI3K), leading to the activation of Akt that regulates cellular processes such as survival, proliferation, and contraction signaling [22]. Integrin-GPCR crosstalk is also linked with the activation of the mitogen activated protein kinase (MAPK) signaling pathway [55]. Lysophosphatidic acid and thrombin receptors alone can activate MAPK in PC12 cells and this was blocked by RGD peptide and cytochalasin D, which is an actin depolymerising agent involved in the remodeling of the cytoskeleton [55]. This data suggests important crosstalk between integrins and GPCRs in regulating MAPK signaling. Amin and coworkers show that β1 integrin plays a crucial role in negating the apoptotic effects of β-adrenergic receptor stimulation in cardiac myocytes via the involvement of FAK and PI3K/Akt pathways [57]. Furthermore, a nonreceptor tyrosine kinase, PYK2, is able to link GPCRs to focal adhesion-dependent ERK activation to provide a point of convergence between signaling pathways triggered by integrins and certain GPCR

Table 1: Expression of different integrins in ASM, their potential ligands and change in expression in asthma.

Integrin	Expression in ASM	Potential ligands	Change in expression in asthma (human)	Reference
$\alpha1\beta1$	Human, sheep, guinea pig	Collagen I, II, III, IV, laminin-111, fibronectin.	n.d.	[27–30]
$\alpha2\beta1$	Human, guinea pig	Collagen I, IV, laminin-111, tenascin.	n.d.	[27–29, 31, 32]
$\alpha3\beta1$	Human	Collagen I, fibronectin, laminin-211, laminin-221, laminin-322, laminin-511, laminin-521.	n.d.	[14, 27, 28]
$\alpha4\beta1$	Human, sheep	Fibronectin, osteopontin, VCAM-1.	↑	[27, 28, 30, 33]
$\alpha5\beta1$	Human, guinea pig	Fibronectin, osteopontin.	↑	[12, 27, 28, 32, 34, 35]
$\alpha6\beta1$	Human	Laminin-111, laminin-411, laminin-511, laminin-521.	n.d.	[14, 28]
$\alpha6\beta4$	Human	Laminin-322, laminin-511, laminin-521.	n.d.	[28]
$\alpha7\beta1$	Human	Laminin-111, laminin-211, laminin-221.	n.d.	[14]
$\alpha8\beta1$	Mouse	Fibronectin, tenascin, vitronectin	n.d.	[28]
$\alpha9\beta1$	Human, guinea pig, mouse	ADAMs 1, 2, 3, 9, 15, factor XIII, L1-Cell adhesion molecule, osteopontin, tenascin, VCAM-1, von Willebrands factor.	↓	[28, 36, 37]
$\alpha v\beta1$	Human	Fibronectin.	↑	[28, 32]
$\alpha v\beta3$	Human	Fibrinogen, fibronectin, GSP, laminin, osteopontin, thrombospondin, vitronectin, von Willebrands factor.	n.d.	[27, 28, 32]
$\alpha v\beta5$	Human, mouse	Osteopontin, vitronectin	↑	[38]

n.d.: not determined.

Table 2: Expression of ECM proteins/integrin ligands, their potential crosstalk with G proteins and change in expression in disease.

ECM/integrin ligands	Potential crosstalk with G proteins	Disease	Reference
Cyr61	$G_{12/13}$	↑ in breast and endometrial cancers	[44]
RGD sequence in $P2Y_2$ receptor	G_0	n.d.	[45]
Laminin-111	G_i, and G_s	↑ in asthma	[46–48]
Fibronectin	G_q and $G_{12/13}$	↑ in asthma	[47–50]
Collagen I	G_q	↑ in asthma	[47, 48, 51]
Collagen V	G_i and G_s	↑ in asthma	[46–48]

n.d.: not determined.

agonists (histamine) in HEK 293 (human embryonic kidney cell line) and HeLa Cells [58]. In another study, Short and coworkers show that the regulation of MAPK activity by integrins and P2Y class of $G_{q/11}$-coupled receptors in human endothelial cells may involve activation of calcium and PKC [55]. Collectively, these studies support a role for integrin and GPCR crosstalk in physiological processes; however, integrin-GPCR interaction may be context-dependent given that different signaling mechanisms have been put forward.

The expression of ECM proteins can be regulated by GPCR ligands. For example, thrombin, sphingosine-1-phosphate, and LPA that signal through $G_{12/13}$ and Rho A activation can increase the expression of the ECM protein Cyr61 (CCN1) in fibroblast, smooth muscle cells, and prostatic epithelial cells, respectively [44]. Cyr61 subsequently binds to integrin and activates downstream signaling pathways that regulate cell migration, survival, and proliferation. The engagement of integrin signaling pathway via GPCR ligands provide a means to amplify and sustain GPCR signaling in normal and pathophysiological cellular functions. In the asthma context, exaggerated GPCR signaling in AHR may contribute to increased expression of ECM proteins in the airway. The activation of integrins by these ECM proteins may thus amplify and sustain GPCR signaling to contribute

to excessive bronchoconstriction that is observed in asthma exacerbations.

Activated integrins organize supramolecular complexes consisting of cytoskeletal domains and associated receptors and signaling molecules that may contribute to the formation of specialized lipid microdomains, which are referred to as "lipid rafts" [59]. Until now, there is no evidence for integrin-mediated activation of heterotrimeric G protein signaling cascade outside lipid rafts. However, there is some evidence to show that ligation of integrins within supramolecular complexes can lead to activation of GPCRs. CD47, an integrin associated protein, forms complexes with $\alpha_V\beta_3$ integrin and activates G_i signaling [60]. Integrin association is also required for activation of G_o signaling by the $P2Y_2$ receptor [45]. Recently, Berg and colleagues show that the relative amount of activated integrins at focal adhesion sites may govern signaling by μ opioid receptor, perhaps by altering interactions with G proteins [17]. Moreover, Lin and coworkers show that integrin ligation can trigger AMPA receptor-dependent Ca^{2+} influx and intracellular Ca^{2+} store release [61]. Taken together, crosstalk between integrins and GPCRs is relevant to ASM cells and possible in the asthma pathophysiological processes.

There are currently limited studies regarding the involvement of both integrins and GPCRs in the regulation of ASM cell contraction in healthy and asthmatic condition (Figure 1). However, crosstalk between integrins and GPCRs in contraction signaling is evident in other cell types. In the context of cardiac muscle contraction signaling, Wang and colleagues show that laminin binding-β_1 integrins in association with the actin cytoskeleton are able to attenuate adenylate cyclase (AC) activity. This in turn inhibits cholinergic regulation of L-type Ca^{2+} current in cardiac muscle contraction [62]. Subsequently, they also show that laminin binding-β1 integrins in conjunction with the actin cytoskeleton have the ability to reduce β1-adrenergic receptor-induced L-type Ca^{2+} and enhance β_2-adrenergic receptor-induced L-type Ca^{2+} current in the same cell [63]. Recently, the same group shows that β_1-integrin-induced activation of the FAK/PI3K/Akt pathway can inhibit β_1-adrenergic receptor-mediated stimulation of L-type Ca^{2+} current in cardiac muscle contraction [64]. This study suggests that increased deposition of ECM proteins such as laminin in a failing heart may favor β_2-adrenergic receptor signaling to β_1-adrenergic receptor signaling, and this may be mediated in part via β1 integrin-induced FAK/PI3K/Akt pathway.

As for atrial myocytes, β_2-adrenergic receptor stimulation of Ca^{2+} current is shown to be enhanced by β_1 integrin via inhibition of cAMP/PKA and activation of G_i/ERK/$cPLA_2$/AA signaling [65]. This study suggests that increased ECM protein deposition in atrial diseases such as atrial fibrosis and/or hypertrophy may enhance β_2-adrenergic signaling, which depends more on G_i/ERK/$cPLA_2$/AA signaling (contraction) instead of G_s/AC/cAMP/PKA signaling (relaxation). Cheng and coworkers also elegantly show the relationship between β_1 integrin and β-adrenergic receptor regulation of L-type Ca^{2+} current in neonatal rat ventricular myocytes [66]. Overexpression of β_1 integrin impedes β-adrenergic receptor-induced Ca^{2+} current via inhibition of AC/cAMP activity [66]. Similar observation is also obtained in adult cat atrial myocytes [62]. These findings suggest an important role for integrin and β-adrenergic receptor crosstalk in a diseased heart in which it is associated with chronic overload of pressure, increased ECM proteins and integrin receptors. Remodeling of GPCR receptor functions in asthma may occur too as there is increased deposition of ECM proteins and altered expression of integrins in the asthmatic airways. Collectively, these studies suggest that integrin activation might play a role in GPCR-induced muscle contraction of the airways.

In the context of ASM cell physiology there is only one study that links ECM proteins to GPCR-induced relaxation signaling [46]. Exposure of ASM cells to laminin decreases cAMP accumulation and AC activity [46]. The decrease in cAMP accumulation and AC activity could be due to a phenomenon known as "G protein switching" [46]. "G protein switching" occurs when agonists binding to the β_2-adrenergic receptor leads to the activation of G_i rather than G_s. The activation of G_i and decreased G_s signaling translate into low AC activity and thus decreased cAMP accumulation [46]. Altered phosphorylation states of the β_2-adrenergic receptor may be the underlying cause of G protein switching [67]. Since integrins are able to phosphorylate cell surface receptors, it is thought to play a role in G protein switching [68]. Human ASM cells predominantly express AC isoforms V and VI. These isoforms can be inhibited by Ca^{2+} and G_i signaling but stimulated by PKC [69, 70]. As integrin activation leads to PKC activation and Ca^{2+} release and influx, it suggests that integrins may modulate AC activity. This would explain the decrease in AC activity of human ASM cells cultured on laminin [71, 72]. This finding is important given that cAMP and AC are regulators of ASM relaxation signaling and this is the first study to implicate that integrins may regulate ASM tone. However, the involvement of GPCR crosstalk with integrins in healthy and asthmatic ASM was not directly investigated in this study and future studies in this area are warranted.

GPCR signaling has been shown to be highly compartmentalized and disruption of this subcellular organization may affect GPCR function [73]. Integrin clustering is a crucial step towards the formation of focal adhesion. Focal adhesion is able to recruit various proteins that are involved in cell signaling cascades which include G proteins in GPCR signaling [74]. Contractile human ASM cells exhibit omega-shaped plasma invaginations known as caveolae (developed from lipid rafts that bind caveolin-1 protein) [75]. Caveolae associate preferentially with signaling proteins that have roles in controlling smooth muscle contraction signaling, for example, G_α protein, members of the Rho small GTPase family, and PKC [75]. Depending on the type of GPCR, upon ligand binding, receptors may remain, exit or translocate into caveolae [76–78]. Muscarinic M_3 and histamine H_1 receptors have been found within the caveolae enriched membrane fraction of human ASM [75]. Moreover, muscarinic M_3 receptors and G_q protein cofractionate in caveolin-1 enriched ASM cell membranes [79]. Caveolin-1 is able to bind to integrin α-subunits and has been shown

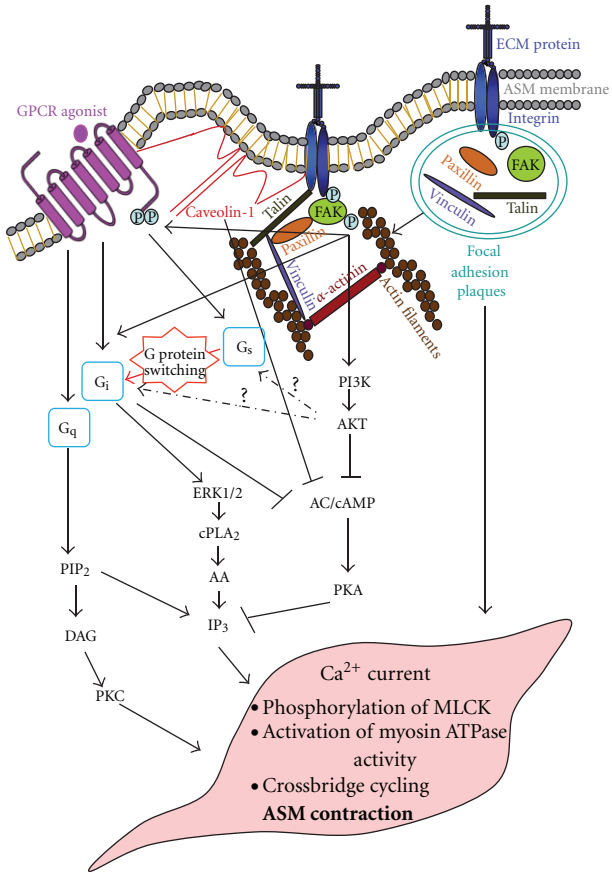

Figure 1: Schematic diagram showing the proposed crosstalk between integrins and GPCRs in ASM cell contraction signaling. Integrin activation is achieved via the formation of focal adhesion plaques leading to cytoskeleton reorganization, which is essential for actin polymerisation and recruitment of linker proteins for tension development. Integrin activation causes the phosphorylation of FAK and activation of downstream signaling events leading to ASM contraction. Integrin activation will also increase intracellular Ca^{2+} concentration to cause phosphorylation of MLCK and activation of myosin ATPase activity and crossbridge cycling. GPCR-induced ASM contraction signaling can be enhanced either by inhibition of cAMP/AC activity that regulates ASM relaxation signaling, or by activation of Ca^{2+} current that is necessary for ASM contraction signaling. Activation of integrins can attenuate GPCR-induced AC activity via the FAK/PI3K/Akt pathway. cAMP accumulation and AC activity can be decreased by integrin activation via G protein switching, in which G_i is activated instead of G_s. Altered phosphorylation of GPCR by integrins is thought to underlie G protein switching in ASM cell. Caveolin-1 that binds integrin has been shown to regulate GPCR signaling. Caveolae which are rich in caveolin-1 function as negative regulators of cAMP accumulation in ASM cell. GPCR stimulation of Ca^{2+} can be enhanced by integrin via inhibition of cAMP/PKA and activation of the G_i/ERK1/2/$cPLA_2$/AA signaling. AA: arachidonic acid; AC: adenyl cyclase; AKT: protein kinase B; ASM: airway smooth muscle; cAMP: cyclic adenosine monophosphate; cPLA2: cytosolic phospholipase A2; DAG: diacylglycerol; ECM: extracellular matrix; ERK1/2: extracellular signal regulated kinase1/2; FAK: focal adhesion kinase; GPCR: G protein-coupled receptor; IP_3: inositol 3,4,5-triphosphate; PIP_2: phosphoinositol 4,5-bisphosphate; PI3K: phosphatidylinositol 3′-kinase; PKA: protein kinase A; PKC: protein kinas C.

to regulate GPCR-mediated signaling [80, 81]. Caveolin-1 links integrin α-subunit to tyrosine kinase Fyn which then recruits Shc and Grb2. This sequence of events couples integrins to downstream signaling pathways such as Ras-ERK pathway. Caveolae function as negative regulators of cAMP accumulation. This suggests that integrin signaling regulated by caveolin-1 may serve as important modifier of GPCR signaling such as cAMP signaling in asthma. Caveolae are found in close proximity to peripheral sarcoplasmic reticulum and mitochondria, suggesting that caveolae may play a role in the spacial coordination of Ca^{2+}-handling channels and organelles, which are implicated in ASM contraction signaling [82]. In addition, caveolae are anchored to the dystrophin glycoprotein complex (DGC). The DGC in turn links to ECM protein, laminin. This linkage is thought to help maintain membrane integrity [75, 83]. Collectively, these studies support the notion that caveolae may mediate ASM contractile response by aiding integrin and GPCR crosstalk signaling in asthma.

Integrins have also been implicated to regulate vascular smooth muscle cell contraction by mobilizing intracellular Ca^{2+}. The addition of RGD peptide at millimolar range elicited increased levels of intracellular Ca^{2+} concentration [18]. This activation of ryanodine-sensitive Ca^{2+} store and lysosome-like organelles by RGD peptide [18, 84] suggests important role of integrin-dependent Ca^{2+} signaling in regulating smooth muscle contraction. In support, $\alpha_7\beta_1$ integrin has been implicated to regulate transient elevation of intracellular-free Ca^{2+} concentration from both IP_3 evoked Ca^{2+} release from intracellular stores and extracellular Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels in skeletal muscle cell [85].

Lastly, it is worth noting that GPCR agonists may promote ECM protein production, either directly, or indirectly by promoting autocrine TGFβ release. TGFβ is linked to thickening of ASM layer and deposition of collagen. Tatler and colleagues show that GPCR agonists, LPA and methacholine, induced TGFβ activation via integrin $\alpha v\beta 5$ by ASM cells [38]. In support, Grainge and colleagues provide evidence that repeated bronchoconstriction with methacholine increases TGFβ immunoreactivity within the airway epithelium and increases the thickness of the subepithelial collagen layer, which is indicative of an acute alteration in airway collagen dynamics [86]. These studies provide alternative means of crosstalk between GPCRs and integrins, and one that could amplify direct GPCR/integrin interactions.

5. Concluding Remarks

In summary, integrins may play a role in regulating GPCR-induced ASM cell contraction signaling in asthma. This finding may offer explanations for increased contractility of ASM cells in asthma in which ECM proteins and their binding receptor integrins are highly expressed. Thus, integrins may be an interesting therapeutic target to inhibit ASM contraction signaling in asthma. However, the development of integrin antagonists has proven to be challenging. The role of integrins in asthma is complex as multiple

integrins may participate to exert asthma symptoms, making it difficult to specifically target integrins that are involved in ASM contraction signaling. Perhaps targeting "linker proteins" that link the crosstalk between integrins and GPCRs in ASM contraction signaling is a possible therapeutic strategy for the treatment of AHR in asthma. One such possible target is caveolin-1 that may regulate integrins and GPCRs crosstalk. Other possible targets may be those which participate in G protein switching that are induced by integrin activation. Nonetheless, further understanding of the mechanisms behind integrin and GPCR crosstalk in ASM cell contraction signaling will enhance the development of more tailored therapy in the future for asthma treatment where AHR is a feature.

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Review Article

Airway Smooth Muscle as a Target in Asthma and the Beneficial Effects of Bronchial Thermoplasty

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Airflow within the airways is determined directly by the lumenal area of that airway. In this paper, we consider several factors which can reduce airway lumenal area, including thickening and/or active constriction of the airway smooth muscle (ASM). The latter cell type can also contribute in part to inflammation, another feature of asthma, through its ability to take on a synthetic/secretory phenotype. The ASM therefore becomes a strategically important target in the treatment of asthma, given these key contributions to the pathophysiology of that disease. Pharmacological approaches have been developed to elicit relaxation of the ASM, but these are not always effective in all patients, nor do they address the long-term structural changes which impinge on the airway lumen. The recent discovery that thermal energy can be used to ablate smooth muscle has led to the development of a novel physical intervention—bronchial thermoplasty—in the treatment of asthma. Here, we review the evolution of this novel approach, consider some of the possible mechanisms that account for its salutary effects, and pose new questions which may lead to even better therapies for asthma.

1. The Airway Lumen: Physiological Importance

The primary function of the lungs is to meet the metabolic demands of the body by absorbing atmospheric oxygen, delivering that to the rest of the body, and excreting carbon dioxide. The importance of this function is reflected in the amount of oxygen required at rest and during exercise. An average 70 kg individual has a resting oxygen uptake of 250 mL/min, which for a number of reasons requires a ventilation rate of 7-8 L/min. First and foremost, alveolar ventilation is very inefficient due to the ventilatory anatomic dead space: that is, the average individual has a resting tidal volume of 300–400 mL (and rate of 15–20 breaths per minute), but gas exchange occurs almost exclusively in the alveoli, with minimal uptake in the conducting airways. Also, it needs to be kept in mind that approximately four fifths of that inspired volume is not useful (atmospheric air is only 20.93% oxygen) and that breathing itself requires effort. If that individual walks briskly at 4-5 km/hour, oxygen uptake goes to 1000 mL/min, requiring about 30 L/min of ventilation. More strenuous exertion (e.g., running up a flight of stairs) can demand ventilatory rates of 125–150 L/min (the metabolic costs are the same for normals and athletes, but the latter are able to reach much higher ventilation rates and power outputs).

Poiseuille's Law relates together the various factors which determine the flow F of a fluid of a given viscosity (η) through a tube (radius r; length L) driven by a pressure gradient ΔP), as follows:

$$F = \frac{(\Delta P \pi r^4)}{(8\eta L)}. \quad (1)$$

A very compelling message to be obtained from this equation is the dramatic effect that narrowing of the vessel lumen has on fluid flow: a change in vessel radius of only 10% can result in a decrease in flow of 36% ($1–0.9^4$), while a narrowing of 20% leads to a 59% reduction in flow ($1–0.8^4$) (this mathematical consideration does not take into account turbulence at the vocal cords and upper airways; elastic and inertial adjustments related to the mass of the chest and abdomen that must also move during breathing, etc.). There

are many factors which can directly influence airway lumen diameter, as outlined in the following.

2. Airway Wall Thickening

Mathematical modelling has shown how changes in airway wall geometry alone—more precisely, thickening of the airway wall—can seriously hinder airflow [1, 2]. The airway wall consists of an epithelial layer founded upon a basement membrane, with a band of airway smooth muscle (ASM) encircling both: changes in all three of these components are known to contribute to wall thickening and, ultimately, to asthma.

2.1. Basement Membrane. The basement membrane and extracellular matrix are made up of various proteoglycans, glycosaminoglycans, and connective tissue proteins (collagen, elastin, fibronectin, etc.). This layer provides structural integrity and a platform on which other cells (epithelium; ASM; inflammatory cells) can reside and/or migrate. More importantly, there is an abundance of studies which have shown increased amounts of connective tissue proteins in the airway wall and underneath the basement membrane layer in asthma; these will not be detailed here but have been reviewed elsewhere [3].

2.2. Epithelial (Goblet) Cell Hyperplasia and Increased Mucous Secretion. An epithelial layer lines the lumenal face of the airways and comprises up to 8 morphologically distinct cell types, the anatomy and functions of which have been described in greater detail elsewhere [4]. However, one of these cell types which is particularly relevant to the current discussion is referred to as the mucous cell or goblet cell. In asthmatics and in many animal models of airway hyperresponsiveness (routinely used to better understand the changes seen in asthma), there is thickening of the epithelial layer due to goblet cell hyperplasia [5, 6]. The latter protrude into the lumen and also secrete a thick mucous: both of these changes lead to an effective decrease in lumenal area (Figure 1(a)) and, thus, to decreased air flow. In some cases, particularly fatal asthma, mucous plugs can completely obliterate airflow.

2.3. Increased ASM Mass. In asthma and in many animal models of airway hyperresponsiveness, the ASM cell layer which encircles the airway wall can also be greatly thickened [7, 8]. There has long been discussion as to whether this is due to hypertrophy (increased cell size) *versus* hyperplasia (increased cell number), with evidence available on both sides of the argument in animal and human preparations [9–11]. However, a recent study which focussed specifically on this question in human airway tissues obtained from normal control volunteers as well as nonfatal and fatal asthmatics concluded that hypertrophy accounts for the thickening in the large airways of both asthmatic groups, while hyperplasia only occurred in fatal asthma [12].

While stereological morphometric and statistical analytical techniques are available to determine whether ASM thickening is due to hyperplasia *versus* hypertrophy, it is not yet possible to determine whether the thickening is primarily directed outwardly (i.e., away from the lumen) and/or inwardly (i.e., towards the lumen) over the months and years as asthma develops. These two changes have very different functional consequences. That is, the resting lumenal area is unchanged in the former case (Figure 1(b)) but markedly decreased in the latter case (Figure 1(c)). In contrast, both changes are associated with increased contractile responses which can also obstruct air flow (next section).

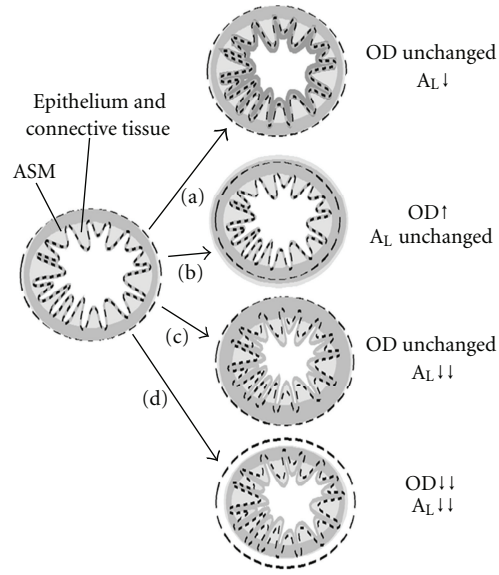

Figure 1: Image on the left depicts a hypothetical airway: dashed lines on the left and right images circumscribe the outer and inner dimensions prior to various changes which can impact airway lumenal area (A_L) and airflow. (a) Thickening of the basal lamina, epithelial cell hyperplasia, edema formation, and/or bronchial vascular dilation all lead to a swelling or thickening of the innermost layer(s) of the airway: outer diameter is unchanged, but A_L is reduced, leading to decreased airflow. On the other hand, the ASM layer itself might become thickened: this can be directed outwardly such that A_L and airflow are unchanged (b) or directed inwardly such that A_L and airflow are both reduced (c). Finally, the ASM can actively constrict, leading to a reduction in outer diameter, A_L and airflow (d).

3. Active Constriction of the ASM

For many decades, the primary function of ASM was seen to be the same as that of all other muscles: to constrict and thereby generate tension and/or shortening. However, over the past decade, questions have been raised as to what would be the physiological purpose of this mechanical response in the airways, and what then is the role of ASM in normal lung physiology [13, 14]; other possible physiological roles for ASM will be mentioned briefly (Section 4) and have been reviewed in more detail elsewhere. Nonetheless, the ASM is able to constrict in response to a wide variety of physiological and pathological stimuli and thereby effect a profound decrease in airway lumen diameter (Figure 1(d)).

The increased ASM mass seen in asthmatics and in animal models of airway hyperresponsiveness (AHR) is expected, then, to lead to increased bronchoconstriction. Increased sensitivity to a variety of inhaled spasmogens (methacholine, histamine, etc.) is well documented in asthmatic individuals [9, 10, 15–22]. However, there is far less consensus as to whether the ASM cell *per se* functions differently in asthma. While many studies using isolated tissues/cells from allergen-induced *animal models* have found larger responses at any given spasmogen concentration (hyperreactivity) and/or a distinct leftward shift in the concentration-response curves for various spasmogens (hypersensitivity) [23–37], studies using *human* airway tissues and cells are far less clear. One study of tracheal smooth muscle obtained from severe asthmatics found increased sensitivity to cholinergic stimulation or to histamine and impaired relaxations to isoproterenol [38], while two other studies using small bronchi from mild [15] or severe [39] asthmatics found no changes in sensitivity to excitatory stimuli (although the potency of isoproterenol was still decreased ~10-fold [39], possibly due to desensitization following frequent use of inhaled β-agonist). Single cells obtained by bronchial biopsy from asthmatic patients showed increased shortening capacity, shortening velocity, and expression of myosin light chain kinase (MLCK) [40]. Bronchial cells cultured from asthmatics show decreased SERCA2 expression and altered Ca^{2+}-handling [41] and retain a hyperproliferative and hypersecretory phenotype through repeated rounds of cell passaging [42]. Thus, there are reasons to question whether basic contractile signalling mechanisms in ASM are altered in asthma.

4. Nonmechanical Functions of ASM

ASM cells are now known to subserve a number of other functional activities, including synthesis and secretion of extracellular matrix proteins and proinflammatory mediators and antigen presentation [43–47]. As such, the ASM itself can contribute in several ways to the wall remodelling and inflammation which characterize asthma; ASM cells from patients with asthma display greater proliferative and synthetic responses compared to ASM cells from nonasthmatic subjects [48]. *In vitro* studies using cultured cells have shown that ASM cells which take on or manifest the secretory phenotype show reduced expression of the contractile proteins α smooth muscle actin and/or smooth muscle myosin. This may explain, in part, why there can be increased ASM mass in asthma but little (or none?) change in the contractile responses of excised tissues. It is unknown whether ASM cells which are able to present antigen also lose the contractile phenotype.

5. The ASM Is a Key Target in Asthma

The symptoms and morbidity associated with asthma are in part a result of episodic bronchoconstriction, which is believed to be largely due to an underlying AHR. One way to quantify pulmonary function is to have the individual inhale fully ("total lung capacity"), then to exhale forcibly and maximally, and measure the volume of air which is expelled within the first second ("forced expiratory volume," or FEV_1). One measure of airway responsiveness to an agonist is to have that individual first inhale an aerosol containing a bronchoconstrictor agent (e.g., methacholine, histamine, etc.) and determine the concentration of agonist which decreases FEV_1 by 20% (PC_{20}). Improvement in asthma severity (e.g., as a result of allergen avoidance [49, 50] or treatment with inhaled corticosteroids [51–53]) is often associated with an improvement of PC_{20} by 1-2 doubling concentrations. These induced changes are small in comparison to the difference in PC_{20} between asthmatic and nonasthmatic populations. Although the magnitude of AHR can fluctuate within an individual, there appears to be limits to the extent to which it can be improved even with optimal disease management based on current guidelines. Moreover, in patients optimally managed under research study conditions, there are reported symptoms on almost 50% of study days, and over 15% of these individuals still experience at least one severe exacerbation per year [54]. Altogether, it is clear that the mechanisms underlying AHR and airway dysfunction are complex and that not all of these are addressed by current treatment practices.

In an attempt to address the aspects of asthma resistant to current therapies, new pharmacological treatments have been developed based largely on our improved understanding of disease mechanisms [55, 56]. Much of the acute and life-threatening changes are due to ASM contraction causing airflow obstruction [38, 57]. Asthmatics carry symptom-relieving inhaled medications, such as a rapidly acting bronchodilator [55, 56, 58], which act within minutes—a timeframe more likely related to muscle relaxation and bronchodilation than to reversal of edema, elimination of mucus, reduction of wall thickening, or relief of any other mechanism by which inflammation causes airway obstruction. These medications are thus extremely valuable for treating asthma but do not modify the disease state: once these medications are stopped, their benefit rapidly wanes. Asthma is also characterized by airway inflammation, to which the ASM also plays a contributory role in that it can synthesize and secrete proinflammatory mediators and present antigen. Altogether, then, the ASM plays a key role in the pathophysiology underlying asthma (while seemingly playing no important or useful role in normal physiology [13, 14]) and is therefore a prime target in our search for better strategies to treat asthma: any approach which selectively decreases ASM mass (and its consequent ability to constrict the airway and contribute to inflammation) could be superior to the current strategy of treating its symptoms. This tantalizing prospect led to ideas of interfering with ASM proliferation or migration, or promoting ASM apoptosis or delivering toxins to the ASM *per se* using immunological or genetic approaches [59]. Thermal energy has been used to reduce smooth muscle mass in other disease states, which led some to try this approach in asthma. In the next two sections, we will summarize that foundational work in non-ASM preparations (Sections 6 and 7), as well as more recent and encouraging data showing its usefulness in ASM and asthma.

6. Response of Smooth Muscle to Thermal Injury

Many have examined the response of smooth muscle to relatively mild heat stress (HS) (<43°C), too numerous to cite here: these describe typical febrile responses including induction of the heat shock protein cascade. However, this level of thermal exposure is not known to be associated with loss of smooth muscle—airway or otherwise.

On the other hand, numerous studies describe the delivery of supraphysiological temperatures (45-65°C) for the treatment of benign prostatic hyperplasia [60–63]. Histological examination of the prostatic tissues weeks after such thermotherapy revealed loss of smooth muscle mass and dark staining of nuclear chromatin, and isolated segments of these tissues showed decreased responsiveness [63]; it is important to note that the degree of these structural and functional changes was not seen at temperatures below 48°C and was thermal "dose" dependent [63]. In another study of the response to HS in the guinea-pig vas deferens, there was loss of adrenoceptors, loss of myofilaments, and dark staining of nuclear chromatin of the smooth muscle cells, again only at temperatures in excess of 50°C [62].

The therapeutic application of HS to coronary arteries has been attempted in the past: "thermal balloon angioplasty" involved introduction of a balloon catheter which was then inflated and heated to temperatures ranging from 50 to 100°C [64–69]. This resulted in reduced vasoreactivity but also produced intimal hyperplasia and fibroproliferation leading to restenosis: this therapy was soon abandoned when it became apparent that the latter long-term structural changes outweighed the short-term functional benefits.

7. Bronchial Thermoplasty

Recently, the direct application of thermal energy was found to be useful in reducing bronchial wall muscle content in asthma [70–75]. While under only local anaesthesia, a four-armed basket electrode is introduced into the airways using a bronchoscopic catheter, expanded to make contact with the airway wall and then used to deliver radiofrequency energy in order to warm the airway wall to a target temperature of 65°C (coffee or tea can be imbibed at higher temperatures than this). The outcome of this procedure—referred to as bronchial thermoplasty (BT)—is an airway which looks normal with respect to epithelium and basement membrane (no evidence of scar tissue) but which now displays approximately 50% less ASM (at least 3–6 weeks following BT) [74] (although it should be pointed out that this study was done in nonasthmatics who were scheduled to undergo lung resection for carcinoma, and it could be argued that inflamed asthmatic ASM may respond differently to HS). Loss of ASM should be associated with reduced potential for bronchoconstriction: indeed, patients with mild, moderate, and severe asthma have been successfully treated, demonstrating persistent improvement in asthma control, better quality of life scores and fewer exacerbations which persist for several years [76–81]. One of the first clinical trials, done in 32 severe asthmatics, found no significant improvement in PC_{20} after 1 year [79], although another larger, 5-year trial with 101 moderate-to-severe asthmatics noted improvement in PC_{20} values in years 2 and 3 [81]; a third 1-year clinical study with 288 severe asthmatics made no comment about PC_{20} values [76]. Also, it has not yet been demonstrated in human lungs that the ASM does not return following BT. The adverse events associated with BT were encountered in the peritreatment period—there were no long-term adverse outcomes such as progressive tissue changes or airway injury. This experience shows that human airways can tolerate thermal interventions, that long-term complications are rare, that benefits can persist for years, and that reduction of smooth muscle content of the airways in humans is safe and feasible.

All of these points notwithstanding, there are limitations associated with BT. First and foremost, there are uncertainties regarding the long-term outcome(s) of this procedure, other than reduction of ASM mass [74]: the cellular/molecular changes which it induces within the lung have yet to be explored in detail. Second, BT is a time-consuming procedure: the bronchial catheter must be inserted into every accessible airway and withdrawn stepwise (in order to allow the basket to make contact with the entire length of each airway) for 10 seconds at each step. There is obviously a considerable degree of invasiveness inherent in this approach, and therefore certain risks are inevitable. Also, this procedure can only be performed on airways which are larger than the diameter of the bronchial catheter, which is unfortunate since smaller airways may also play a role in clinically relevant resistance to airflow.

8. How Does BT Work?

The response to HS is a phylogenetically ancient cellular response to exogenous stress (including high temperatures) which leads to binding of HS-activated transcription factors to cis-acting HS-response elements found within the promotor regions of a number of genes, including HSPs [82] and cytokines [83]. HSPs have been found in every species in which they have been sought [82]. In general, they function as molecular chaperones, binding to proteins which are not in their native conformation due to denaturing stresses (such as heat) or which are not yet fully modified following *de novo* synthesis and thus restore function. They can also contribute to regulation of the intracellular matrix, as they have recently been shown to do in ASM stimulated with physiological autacoids [84, 85]. Generally speaking, HSPs are taken to be beneficial and/or protective in nature. However, others have shown they can also exacerbate injurious events: for example, while febrile-range hyperthermia accelerated pathogen clearance and increased survival in experimental *Klebsiella pneumoniae* peritonitis, it also accelerated lethal lung injury in a mouse model of pulmonary oxygen toxicity, leading the authors to suggest that the lung may be particularly susceptible to the injurious effects of hyperthermia [86, 87]. Likewise, there can be a bimodal effect of HSPs, with decreased protection or even deleterious effects when HSPs

are overexpressed [82] (as would occur following such a severe thermal injury). Febrile-range hyperthermia (~42°C, 120 min) of cultured airway epithelial cells triggers an HSP response followed by increased expression of interleukin-8/CXCL-8 [88, 89].

On the other hand, it may be that HS upsets the balance of removal and renewal of the ASM, by triggering some kind of apoptotic response, or disrupting ASM proliferation and/or migration. We have examined the cellular structural responses to HS caused by brief (30–60 seconds) immersion of excised ASM tissues into a physiological buffer medium warmed to various temperatures as a surrogate for BT. Using TUNEL and immunohistochemical analytic techniques (for DNA laddering and caspase 3 activation), we found significant cell death in all tissues heated to 65°C and limited cell death at lower temperatures. Similarly, NADH diaphorase activity (another measure of cell death) was reduced at 55°C and abolished at higher temperatures.

Prior to that study, we had used the thermal immersion model of BT to study the immediate effects on ASM mechanical function [90]. We found that contractile responses to millimolar potassium chloride or acetylcholine could be completely abolished within seconds of treatment using 55°C or higher, whereas temperatures of 50°C or lower were relatively inconsequential (unless much longer treatment durations were employed). The rapid onset of this response to heat treatment (timeframe of seconds) ruled out the interpretation that the apoptotic cascade or heat shock proteins are involved in mediating the loss of contractility, since both processes require expression of various proteins (timeframe of many hours). Instead, our data supported the interpretation that the immediate loss of functionality was due to denaturation of the myosin molecules, possibly preventing ATP hydrolysis and/or their interaction with actin.

Other temperature-sensitive mechanisms may be involved in the response to BT. One subclass of transient receptor potential (TRP) channels is activated by warmth (25–40°C; TRPV3 and TRPV4), febrile temperatures (>43°C; TRPV1 [91]), or noxious heat (>52°C; TRPV2 [92]). Involvement of one or more of these would be evidenced by a very sharp thermal sensitivity profile and short latency (on the order of milliseconds). TRPV1 and TRPV2 channels have indeed been identified in ASM [93], although there is no clear *a prior* reason to expect that their activation would lead to loss of mechanical function.

On the other hand, a much earlier study found MLCK to be irreversibly inactivated by Ca^{2+}/calmodulin-dependent kinase II (CamK-II) in a steeply temperature-dependent manner, with half-maximal effect at ~55°C when $[Ca^{2+}]$ in the enzyme reaction assay was high (lower temperatures were effective at lower $[Ca^{2+}]$) [94]. Inactivation of MLCK could easily account for loss of mechanical function.

BT may also be reducing the amount of vascular smooth muscle mass in the airway wall: there is some evidence that vascularization of the airway wall is increased in some forms of asthma [95, 96], and others have suggested that dilation of that vascular bed contributes in part to asthma induced by exercise or inhalation of cool, dry air.

Finally, it may be that BT leads to a functional denervation. The earliest histological data obtained during the development of BT as a clinical tool reported that the airway wall a few weeks following thermal injury appeared normal apart from a notable reduction in ASM fibers: the epithelium appeared to be unaffected (although this may have been repopulated by new/other cells). However, neither histological nor functional studies were performed to determine whether nerve fibers and varicosities were also normal. This too is relevant in that (i) the ASM receives strong excitatory neural input from the cholinergic innervation, and cholinergic receptor antagonists (atropine; ipratropium; tiotropium) have proven to be useful in asthma; (ii) sensory neuronal axon reflex can lead to powerful bronchoconstriction [97].

9. Conclusion and New Directions

Altogether, then, studies using animal tissues which had been immersed in heated medium to induce HS suggest that BT in humans may be associated with an immediate (within seconds) loss of ability on the part of the ASM to generate a mechanical response, induction of cell death over the next 1–24 hours, and a marked reduction in ASM mass over the ensuing weeks and months. The net result is expected to be widening of the airways and lessened ability of the airways to actively constrict (although this has not been conclusively demonstrated in humans), thereby removing those geometrical burdens on airflow and possibly also a lessening of the airway inflammation (since the ASM can be a source of inflammatory mediators) which accompanies and exacerbates asthma. More importantly, there is an improvement in quality of life scores, and fewer exacerbations.

The precise mechanism(s) by which BT produces its beneficial effects are far from clear and require extensive further studies. In particular, the exact pathway(s) that lead to loss of airway smooth muscle—various apoptotic responses, autophagy, necrotic cell death, and so forth—are far from clear. A better understanding of these questions may lead to enhancements in the delivery of the thermal injury or, even better, substitution of this physical approach with some form of chemical/pharmacological agent which will trigger those pathways directly. The latter may be better in that they might be easily inhaled, and it would then be possible to ablate ASM in all the airways (large and small; upper lobes as well as lower lobes), rather than just a large fraction of the ones which are easy to reach with a bronchoscope.

Numerous other interesting and imperative questions remain. Why does the ASM layer not return following BT? Does BT somehow alter the airway wall, particularly the connective tissue matrix, such that new ASM cells do not migrate in? Does it somehow alter the remaining ASM cells which survive BT such that they do not proliferate? If so, how? What effects does BT have on the innervation? On fibroblasts?

Answers to these questions may eventually lead to an actual "cure" for asthma, rather than the current approaches

which seek to treat its symptoms (bronchodilators and anti-inflammatories).

Abbreviations

A_L:	Airway lumenal area;
AHR:	Airway hyperresponsiveness;
ASM:	Airway smooth muscle;
BT:	Bronchial thermoplasty;
CamK-II:	Ca^{2+}Calmodulin-dependent kinase II;
ΔP:	Pressure gradient;
F:	Flow;
FEV_1:	Forced expiratory volume;
HS:	Heat stress;
HSP:	Heat shock proteins;
L:	Length (of a tube);
MLCK:	Myosin light chain kinase;
η	Fluid viscosity;
PC_{20}	Concentration of agonist which decreases FEV_1 by 20%;
r:	Radius;
TRP:	Transient receptor potential.

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Research Article

Substance P Regulates Environmental Tobacco Smoke-Enhanced Tracheal Smooth Muscle Responsiveness in Mice

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Environmental tobacco smoke (ETS) is an environmental trigger that leads to airway inflammation and airway hyperresponsiveness (AHR) in susceptible individuals and animals, but the underlying mechanism is not fully understood. Substance P (SP) release from sensory nerve fibers has been linked to AHR. The present experiments characterize the role of SP in tracheal smooth muscle on ETS-increased airway responses. The mice were exposed to either sidestream tobacco smoke (SS), a surrogate to ETS, or filtered air (FA) for 1 day or 5 consecutive days. Contractions of tracheal smooth muscle to SP and electrical field stimulation (EFS) were not significantly altered in 1 of day SS-exposed mice. However, 5 of days SS exposure significantly increased airway smooth muscle contractions to SP and EFS. Administration of CP-99994, an antagonist of the neurokinin (NK)1 receptor, attenuates the SS exposure-enhanced tracheal smooth muscle responses to EFS. Furthermore, the immunohistochemistry showed that SP nerve fibers were increased in tracheal smooth muscle after 5 of days SS exposure. These results suggest that the increased SP production may contribute to SS-enhanced smooth muscle responsiveness in mice trachea.

1. Introduction

Environmental tobacco smoke (ETS) is an environmental trigger that leads to airway inflammation and airway hyperresponsiveness (AHR) in susceptible individuals and animals [1–5]. Epidemiological studies show that the probability of developing or exacerbating asthma increases in ETS exposure patients [3–10]. But the underlying mechanism initiated by ETS exposures that affect lung function remains undefined.

The nervous system, including the nerves supplying the airways, is highly susceptible to environmental influences [11]. The airways are innervated through autonomic and sensory nerve fibers [12, 13]. Sensory innervation in the airways is important in the pathogenesis of inflammation associated with asthma. Substance P (SP), a member of the tachykinin family, releases from sensory nerve fibers and has potent effects on airway smooth muscle tone, vascular permeability to protein and mucus secretion [14, 15]. Recent studies have shown that SP plays an important role in antigen- and irritant-induced AHR [2, 16, 17]. Furthermore, the studies including ours found that increased SP in the airway is involved in cigarette smoke exposure-induced AHR and airway inflammation [2, 18–25]. However, the role of SP in cigarette smoke exposure-enhanced airway responsiveness is not clear.

Recent studies found that SP also acts as a neuromodulator increasing the tracheal smooth muscle contractions to electrical field stimulation (EFS) [26–30]. Both of Watson and Hall's studies found that exogenous and endogenous SP facilitated EFS-induced tracheal contractions, but had no effect on contractions induced exogenous ACh [26, 27]. Nadel's group further showed that SP potentiated airway smooth contraction to EFS in a concentration-dependent fashion [28, 29]. Tournoy et al. also found that EFS-induced airway smooth muscle contraction was decreased in NK1 receptor knockout mice compared with wild-type control [30]. All of these studies suggest that SP enhances EFS-induced airway smooth contraction due to increasing the release of ACh (Figure 1). Thus, we hypothesized that ETS exposure increases SP from sensory nerves or changes NK

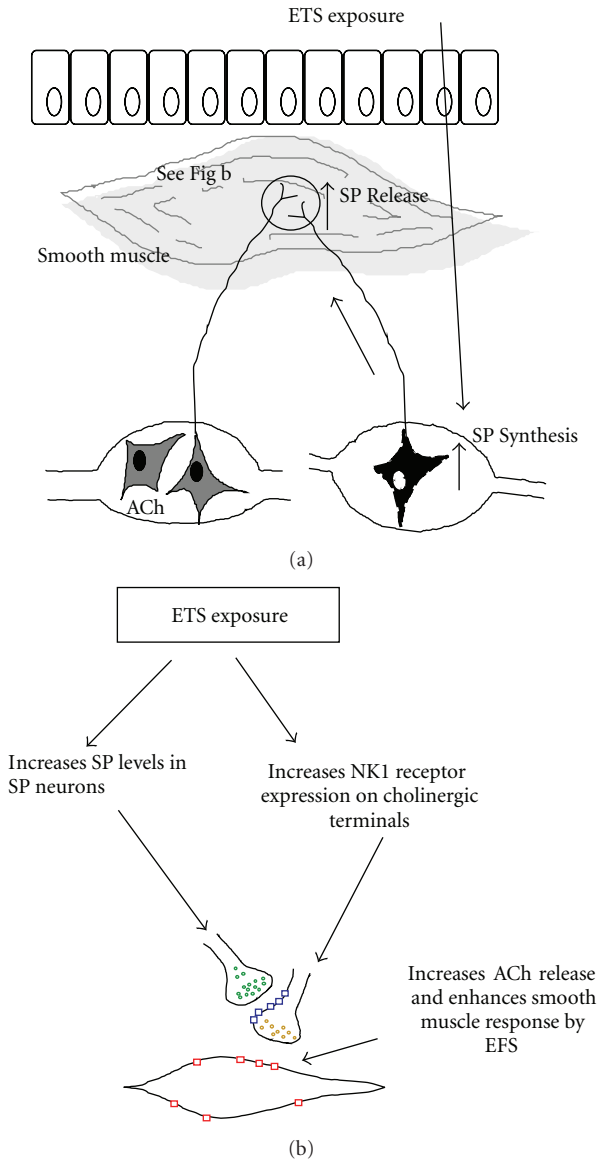

Figure 1: The diagrams demonstrate the effect of increasing SP and NK1 receptor expression on cholinergic nerve of airway smooth muscle. The airway smooth muscles are innervated by cholinergic and sensory nerve fibers (a). The enhancing SP associates with NK1 receptor on the surface of cholinergic neurons, which alters the sensitivity of cholinergic neurons or activates cholinergic neurons and facilitates ACh release from cholinergic nerve (b).

receptor, which facilitates ACh release from cholinergic nerve and enhances airway smooth muscle responses (Figure 1). The enhanced airway smooth muscle response may contribute to increased susceptibility to AHR. The present experiments characterize the role of SP on ETS-enhanced airway smooth muscle responses.

2. Material and Methods

ICR mice (Harlan, Indianapolis, IN, USA) were housed with access to food and water ad libitum in an FDA-approved facility. All procedures were performed in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, and the protocols were approved by the WVU Animal Care and Use Committee no. 05-0503. The animals were treated humanely and with regard for alleviation of suffering.

2.1. SS Exposure. By classical definition, environmental tobacco smoke (ETS) is a diluted mixture of the smoke given off by the burning end of a tobacco product (side stream smoke, ~85%) and the smoke exhaled by smokers (mainstream smoke, ~15%). Based on previous ETS exposure studies [1, 31, 32], we used sidestream tobacco smoke (SS) as a surrogate to ETS.

The two-month-older mice were exposed to either SS or filtered air (FA) for 6 h/day for 1 day or 5 consecutive days. The trachea and lung tissues were collected 16 h after SS or FA exposure. A major goal of the current study was to understand possible neuronal mechanisms by SS that affect lung function. The SS exposure protocol and methods used in this study have been described in our recent publication [1]. Briefly, mice were randomly placed in an exposure chamber (BioClean, DuoFlo model H 5500, Lab Products Inc) that measured $1.92 \times 1.92 \times 0.97$ m ($3.58\,m^3$). The mice were housed in separate cages located in the exposure chamber. Sidestream smoke from Marlboro filter cigarettes (Phillip Morris, Richmond, VA, USA) was introduced into the exposure chamber at a rate of four cigarettes every 15 minutes for 6 hours using a smoking machine (RM 1/G, Heinr Borgwaldt GmbH, Hamburg, Germany). At the end of the 6 h exposure period, the exhaust fan unit was turned on to rapidly lower the level of smoke in the exposure chamber. The mice were then transported to the animal facilities overnight. The concentrations of carbon monoxide in the exposure chamber were monitored and kept to an average of about 50 parts per million (ppm), relative humidity was about 50%, and temperature was about 23°C. Total suspended particulate concentration was about $1.1\,mg/m^3$, similar to exposure levels used by others to approximate the cloud of particulates surrounding a person during active smoking [32].

2.2. Measurement of Tracheal Smooth Muscle Contraction. Fresh tracheas from mice 16 h after FA or SS exposure were cut into 3 mm wide rings, mounted in holders, and maintained in gassed ($95\%O_2$-$5\%CO_2$) modified Krebs-Henseleit solution at 37°C with a composition (in mM) as follows: 113 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 24 $NaHCO_3$, 1.2 KH_2PO_4, and 5.7 glucose, pH 7.4. Each tracheal ring was loaded into a pair of stainless-steel hooks, suspended in tissue holders. Each holder was anchored in a 10-mL water-jacketed organ bath, and the top steel hook was attached to a force-displacement transducer connected to a recorder (Gould Instruments, Valley View, OH, USA). The rings were equilibrated for 60 min at a resting tension of 0.25 g, determined by preliminary studies, during which time the modified Krebs-Henseleit solution in the baths was changed every 15 min. After equilibration, methacholine

(MCh) responses were constructed by adding 10^{-5} M MCh to the bath and SP cumulative concentration-response curves were constructed by adding a series of concentrations of SP to the bath in half-log-increment concentrations ranging from 10^{-9} to 10^{-5} M. After concentration-response curves were completed, electrical-field-stimulation- (EFS-) induced responses were obtained with a stimulator (model S48, Grass Instruments, Richmond, VA, USA). Frequency-response curves were constructed by increasing the frequency from 0.3 to 30 Hz using a submaximum voltage of 120 V, 0.2-ms pulse duration, and 10 s train duration. Between each stimulation period, 10 min were allowed for the previous response to return to baseline. EFS-induced contractions were normalized by the percent response of each tissue to 10^{-5} M MCh. In some experiments, atropine (10^{-6} M) was added to Krebs solution to verify that the responses elicited by EFS were mediated by the release of ACh. To determine the possible involvement of endogenously released SP, some experiments were given CP-99994 (final concentration 10^{-6} M), SP receptor antagonist, which was incubated at least 30 min before addition of SP or EFS. The dose of this antagonist was determined by dose-response curves.

2.3. Immunohistochemistry. The procedures for immunohistochemical quantification of airway nerves have been described previously [1, 33, 34]. Briefly, in a separate group of mice-exposed using the same SS exposure protocol, tracheas were removed 16 h after SS or FA exposure, fixed in picric acid-formaldehyde fixative for 3 h, and rinsed three times with a 0.1 M phosphate-buffered saline containing 0.15% Triton X-100. The tracheas were dissected and frozen in isopentane, cooled with liquid nitrogen, and stored in airtight bags at −80°C. The tracheas were oriented with the dorsal surface uppermost so the tracheal muscle would be sectioned in a coronal plane.

Cryostat sections (12 μm thick) were collected on gelatin-coated coverslips and dried at room temperature. The cryostat sections on coated coverslips were covered with SP antibody diluted 1 : 100, incubated at 4°C for overnight, and rinsed with a 1% bovine serum albumin + phosphate-buffered saline containing 0.15% Triton X-100 three times, with 5 min being allowed for each rinse. The sections were then covered with fluorescein isothiocyanate-labeled goat antirabbit antibody diluted 1 : 100, incubated at 37°C for 45 min, and rinsed. After all immunohistochemical procedures were conducted, the coverslips were mounted with Fluoromount and observed with a fluorescence microscope equipped with fluorescein (excitation wavelengths 455–500 nm, emission wavelengths > 510 nm). Controls consisted of testing the specificity of primary antiserum by absorption with 1 μg/ml of the specific antigen. Nonspecific background labeling was determined by omission of primary antiserum.

For measuring nerve fiber density in tracheal smooth muscle, we collected images of control, SP nerve fibers in series under the Zeiss LSM 510 confocal microscope. A series of images representing all of the tracheal smooth muscles in a section was collected in digital files and saved to an internal database and measured using Optimus software. We selected regions of smooth muscle using the rhodamine channel to avoid possible bias created by the presence or absence of nerve fibers. The smooth muscle regions were outlined to measure total cross-sectional area of smooth muscle. The microscope was then switched to reveal nerve fibers in the fluorescein channel, and the image was digitally captured. The threshold levels were manually adjusted to subjectively optimize the appearance of fluorescent nerves. The area of nerve fibers was determined by segmentation with the Optimus software. Then nerve fiber area was standardized to the total cross-sectional area of smooth muscle. The final value of nerve fiber density is expressed as % of dividing the SP nerve fiber area by the total area of smooth muscle. At least 10 measurements were made for each section, and 15 sections were measured in each animal.

2.4. SP Enzyme-Linked Immunosorbent Assay. SP ELISA assay in lung tissue was conducted as in our previously described work [1]. Lungs were obtained from each animal 16 hours after SS exposure. The specimens were weighed, homogenized, and centrifuged (40,000 g). Supernatant fractions were collected, filtered, and frozen at −80°C until assay. The concentration of SP (39–2500 pg/mL) in each sample was assayed using the SP immunoassay system (R&D systems, Minneapolis, MN, USA) according to manufacturer's instructions. All samples were running in duplicate.

2.5. Data Analysis. Unless otherwise stated, values are means ± SE. Contractions elicited by EFS and SP are expressed as a percentage of the contraction elicited by MCh 10^{-5} M. The half-maximal effective concentration (EC_{50}) values for SP were calculated using a four-parameter logistic curve fit (Sigmoidal, SigmaPlot 2000) and were presented with 95% confidence intervals. Force development was expressed by normalizing force (g) divided by the wet weight of the tissue. Nerve fiber density was expressed as % area of SP-immunoreactive nerve fibers in the total area of the smooth muscle. Statistical analyses of immunohistochemistry and EC_{50} were performed using Student's t-test. Statistical analysis of EFS was performed using two-way repeated-measures analysis of variance. One factor between the groups was SS exposure; the other factor within the group was EFS or SP effect. When the main effect was considered significant at $P < 0.05$, pairwise comparisons were made with a post hoc analysis (Fisher's least significant difference). $P < 0.05$ was considered significant, and n represented the number of animals studied.

2.6. Materials. MCh chloride and atropine sulfate were obtained from Sigma Chemical (St. Louis, MO, USA). CP-99994 was obtained from Pfizer (Groton, CT, USA). SP and SP antibody were obtained from Peninsula (Belmont, CA, USA). Fluorescein isothiocyanate-labeled goat antirabbit antibody was obtained from ICN Immunobiologicals (Costa Mesa, CA, USA).

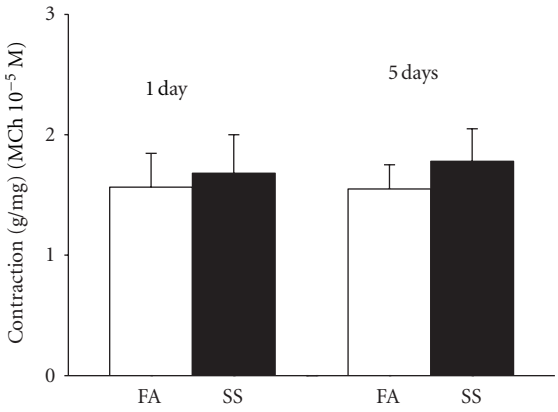

Figure 2: Tracheal smooth muscle contraction was generated by 10^{-5} M methacholine (MCh) in 1 day ($n = 16$ in each group) and 5 days ($n = 30$ in each group) SS-(solid bars) or FA-(open bars) exposed tracheal rings. Values are means ± SE.

3. Results

3.1. Effect of SS on Airway Responsiveness. The initial experiments were intended to find if SS exposure increases airway smooth muscle contraction to MCh. There was no significant difference in the tracheal smooth muscle contraction to MCh (10^{-5} M) between 1 day-SS-exposed ($n = 16$) mice and FA exposed mice ($n = 16$) (Figure 2). Although 5 days of SS exposure slightly increased smooth muscle contraction to MCh, there was no significant difference in airway responses to MCh between 5 days of SS-exposed ($n = 30$) mice and FA exposed mice ($n = 30$) (Figure 2).

The next experiments were intended to figure out the effect of SS on airway responsiveness to SP and EFS. The cumulative dose-response curve for SP was not significantly shifted to the left after 1 day of SS exposure (Figure 3(a)). There is no significant difference between EC_{50} values in SS exposure (6.17 ± 0.11) and FA exposure (5.96 ± 0.14). Also the frequency-response curve to EFS was not significantly altered 1 day of SS exposure (Figure 3(b)). However, the cumulative dose-response curve for SP was markedly shifted to the left after 5 days exposure to SS (Figure 3(c)), there is significant difference between EC_{50} values in SS exposure (6.65 ± 0.09) and FA exposure (6.01 ± 0.11). Also, a leftward shift in the frequency-response curve to EFS was observed after 5 days of SS exposure (Figure 3(d)), and contractions produced by EFS at 10 Hz and 30 Hz were significantly increased after 5 days of SS exposure. The pretreatment with atropine (final concentration 10^{-6} M) totally abolished EFS-induced airway smooth muscle contraction in both FA and SS-exposed mice (Figures 3(b) and 3(d)), indicating that EFS-induced airway smooth muscle contraction mainly involves endogenously acetylcholine (ACh) release.

3.2. Effects of NK1 Antagonist on 5 Days of SS-Enhanced Airway Responsiveness. To ensure sufficient reduction of SP effects, the effective dosage of NK1 receptors antagonist, CP-99994, was tested first. In separate experiments, the different concentrations of CP-99994 were tested by SP cumulative doses (10^{-9} M to 10^{-5} M). The SP cumulative dose-response curves were significantly shifted to the right in a concentration-dependent manner by CP-99994 (Figure 4). EC_{50} values for SP are 6.04 ± 0.14 (control), 5.83 ± 0.12 (CP-99994 10^{-8} M), 5.64 ± 0.11 (CP-99994 10^{-7} M), and 5.56 ± 0.11 (CP-99994 10^{-6} M). There is significant difference in EC_5 between control and CP-99994 10^{-6} M. The combination of CP-99994 (10^{-6} M) and NK2 receptors antagonist SR48968 (10^{-6} M) virtually abolished SP-induced airway smooth muscle contraction, indicating that CP-99994 10^{-6} M is effective dose to block SP-induced airway smooth muscle contraction. Thus, next experiment, CP-99994 10^{-6} M was chosen to test the role of SP on 5 of days SS-enhanced airway smooth muscle responses.

CP-99994 (final concentration 10^{-6} M) was incubated at least 30 min before using SP or EFS. Cumulative concentration–response curve for SP and the EFS-stimulated contractions in saline pretreated groups after 5 of days SS exposure (Figures 5(a) and 5(c)) demonstrated similar changes as those described above, 5 days of SS exposure. The contractions produced by EFS at 10 Hz and 30 Hz were significantly increased in saline pretreated groups after 5 days of SS exposure. CP-99994 did not significantly affect EFS-induced contraction in FA-exposed animals. The tracheal smooth muscle contractions to EFS at 10 and 30 Hz were not significantly altered in the CP-99994 treatment group (10 Hz: 21.44 ± 2.54%; 30 Hz: 25.8 g ± 2.31%) compared with the saline group (10 Hz: 23.36 ± 2.28%; 30 Hz: 27.89 ± 2.29%) in FA exposed mice. However, CP-99994 significantly inhibited the SS-enhanced smooth muscle responses to EFS at 10 and 30 Hz (Figure 5(d)). There are no significant differences in EFS-induced tracheal smooth muscle contractions between 5 of days SS-exposed mice and FA exposed mice after CP-99994 treatment. Also, the enhanced airway responses to SP were total abolished by CP-99994 treatment (Figure 5(b)).

3.3. Effect of SS on Changes of SP in Trachea and Lung. SP nerve fibers in the tracheal smooth muscle of mice were analyzed based on the immunohistochemical localization by fluorescein. The SP nerve fibers were mainly localized on tracheal smooth muscle and epithelium (Figure 6(a)). There is no significant difference in density of SP nerve fibers in the airway smooth muscle between 1 day of SS exposure and FA exposure (Figures 6(a) and 6(b)). However, the density of SP nerve fibers in the airway smooth muscle was significantly increased after 5 days of SS exposure (Figures 6(a) and 6(b)). These findings suggest that 5 days of SS exposure increases SP content in tracheal smooth muscle. Furthermore, the SP level in lung was measured using ELISA. ELISA data showed that SP levels in lung were significantly elevated in 5 days of SS-exposed mice compared with FA exposure (Figure 6(c)).

4. Discussion

The results obtained from the current study show that exposure to SS significantly enhances tracheal smooth muscle responsiveness in the mice, as evidenced by elevated contractility to SP and EFS. The elevation of airway smooth muscle

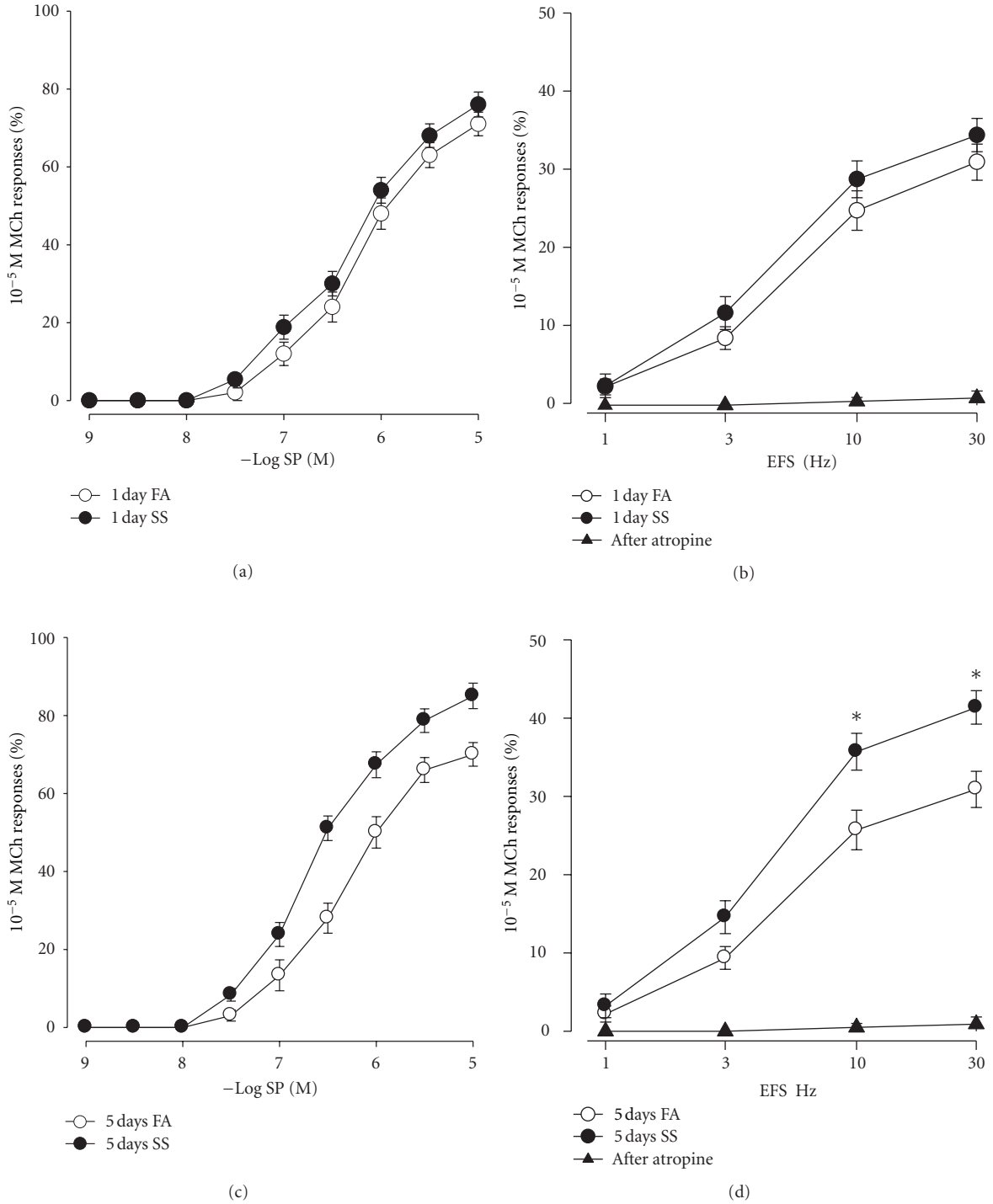

Figure 3: Cumulative concentration-response curves for SP (a, c) and frequency-response curves for electrical field stimulation (EFS; b, d) in tracheal smooth muscle after 1 day (a, b) and 5 days (c, d) exposure to FA (open circles) or SS (circles). The solid triangle showed that atropine (final concentration 10^{-6} M) totally abolished EFS-induced airway smooth muscle contraction. Responses to SP and EFS are plotted as a percentage of the 10^{-5} M MCh response. Values are means ± SE; $n = 16$ in each group. *Significant difference in EFS between FA and SS exposure, $P \leq 0.05$.

responses by SS exposure was attenuated by treatment with a NK1 receptor antagonist, indicating that SP played a key role in the enhancement of smooth muscle contractile responses. Our previous study showed that exposure to irritant changes SP airway innervation and enhances tracheal smooth muscle responsiveness [25, 35], suggesting that SP may contribute to irritant-enhanced smooth muscle responsiveness. The current results from immunohistochemistry found that SS

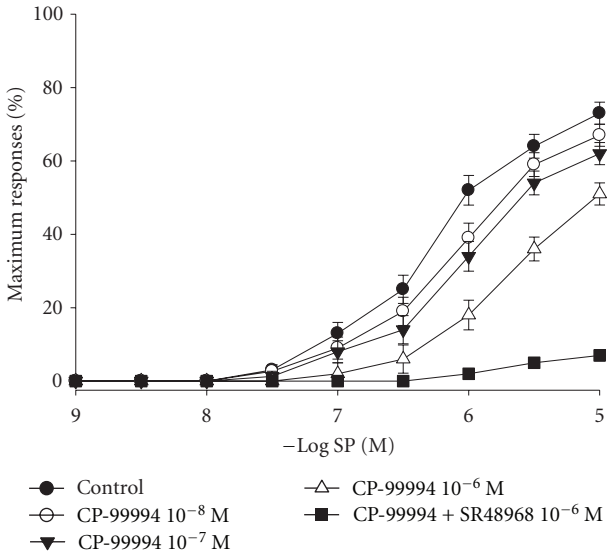

Figure 4: The effect of different concentrations of CP-99994 on cumulative concentration-response curves for SP in isolated mice tracheal smooth muscle. Values are means ± SE, $n = 6$ in each group.

exposure changes SP innervation of tracheal smooth muscle, directly indicating that increased SP level in airway involved SS-enhanced smooth muscle responsiveness.

Airway sensory nerves play a central role in airway regulation [36]. SP localized in the peripheral endings of sensory nerves innervating the lung and airways originates in nerve cell bodies located in sensory ganglia [37, 38]. Stimulation of sensory nerves by inhalation of irritants is known to trigger the release of SP from afferent endings [2, 25, 39, 40]. The data in the present study found that SS-enhanced-airway smooth muscle contractions to EFS were attenuated by SP receptor antagonist, directly indicating that SP is involved. However, the role of SP in smoke exposure-enhanced airway responsiveness is not clear. Recent studies found that SP acts as a neuromodulator altering airway responsiveness to EFS [26–30]. Watson and Hall's studies found that exogenous and endogenous SP facilitated electrical field stimulation (EFS)-induced tracheal contractions, but had no effect on contractions induced exogenous ACh [26, 27]. Nadel's group further showed that SP potentiated airway smooth contraction to EFS in a concentration-dependent fashion [28, 29]. Thus, one possible explanation for our finding is that enhanced SP in airway after SS exposure acted as mediator, which altered airway responsiveness to EFS. Our result found that administration of atropine completely blocks airway smooth muscle responsiveness to EFS in both FA and SS-exposed animals, demonstrating that smooth muscle contraction to EFS in mice trachea is entirely atropine sensitive and totally depends on endogenous ACh release from parasympathetic nerve terminals. It also indicates that there is no SP release in EFS-induced airway responses. Furthermore, our MCh experiments found that SS exposure did not increase airway contractions to MCh and suggested enhanced sensitivity of airway smooth muscle was not involved in SS exposure. Thus the logical explanation is that enhanced SP acts as a neuromodulator increasing the release of acetylcholine (ACh) from parasympathetic nerve. Although the exact mechanism of enhanced ACh release from parasympathetic nerve was not determined in the present study, the finding that the SP receptor antagonist CP-99994 significantly attenuated the effect of SS on EFS responses in trachea suggests the idea that SS exposure induces SP upregulation. The enhancing SP may associate with NK1 receptor on the surface of cholinergic neurons, which alters the sensitivity of cholinergic neurons or activates cholinergic neurons and facilitates ACh release from cholinergic nerve.

Our data also found that contractions of airway smooth muscle to EFS at 10 and 30 Hz were slightly decreased in the CP-99994 treatment group (10 Hz: 21.44 ± 2.54%; 30 Hz: 25.89 ± 2.31%) compared with the saline treatment group (10 Hz: 23.36 ± 2.28%; 30 Hz: 27.89 ± 2.29%) after FA exposure. It indicates that normal level of SP may involve airway responses to EFS. Tournoy et al. study that EFS-induced airway smooth muscle contraction was decreased in NK1 receptor knockout mice compared with of NK1 receptor wild-type controls [30] supports that normal level of SP in airway smooth muscle involved airway responses to EFS, although there is no direct effect of SP on EFS-induced airway responses.

The present experiment also showed that SP dose responses are enhanced after 5 days SS exposure. One possibility is that sensitivity of airway smooth muscle to SP is enhanced by SS exposure. However, it is unlikely that the sensitivity of airway smooth muscle was enhanced due to sensitivity of airway smooth muscle to MCh that was not significantly changed. Alternatively, an altered density and/or expression of the NK receptors or properties of NK receptors (e.g., affinity) on the airway smooth muscle may be involved by SS exposure. Our experiments used three concentrations of CP 99994 and detected that even high-dose CP 99994 (1 μM) only partially but incomplete inhibited exogenous SP-induced airway contraction, suggesting that mice airway may contain a heterogeneous population of receptors. Furthermore, our experiment uses combination with NK1 and NK2 receptor antagonist (SR 48968) treatment and found that combination with NK1 and NK2 receptor antagonist treatment virtually eliminated the response to SP in airway, indicating both NK1 and NK2 receptors mediate the contractile response to SP. Thus, the possible explanation is that SS exposure alters density and/or expression of the NK receptors or properties of NK receptors (e.g., affinity) on the airway smooth muscle, which affect airway repose to SP.

The previous studies including ours have found that childhood exposure to ETS is a significant risk factor for exacerbation of asthma, but studies of smoking in adults are less conclusive. There are evidence that mainstream cigarette smoking exposure may decrease the incidence of some chronic inflammatory and have a beneficial effect of smoking on airway responsiveness [41–44]. These studies found that exposure to mainstream cigarette smoke attenuates airway responses to ACh, methacholine [41–43] and neurokinin A [44], also decreased airway inflammation comparing

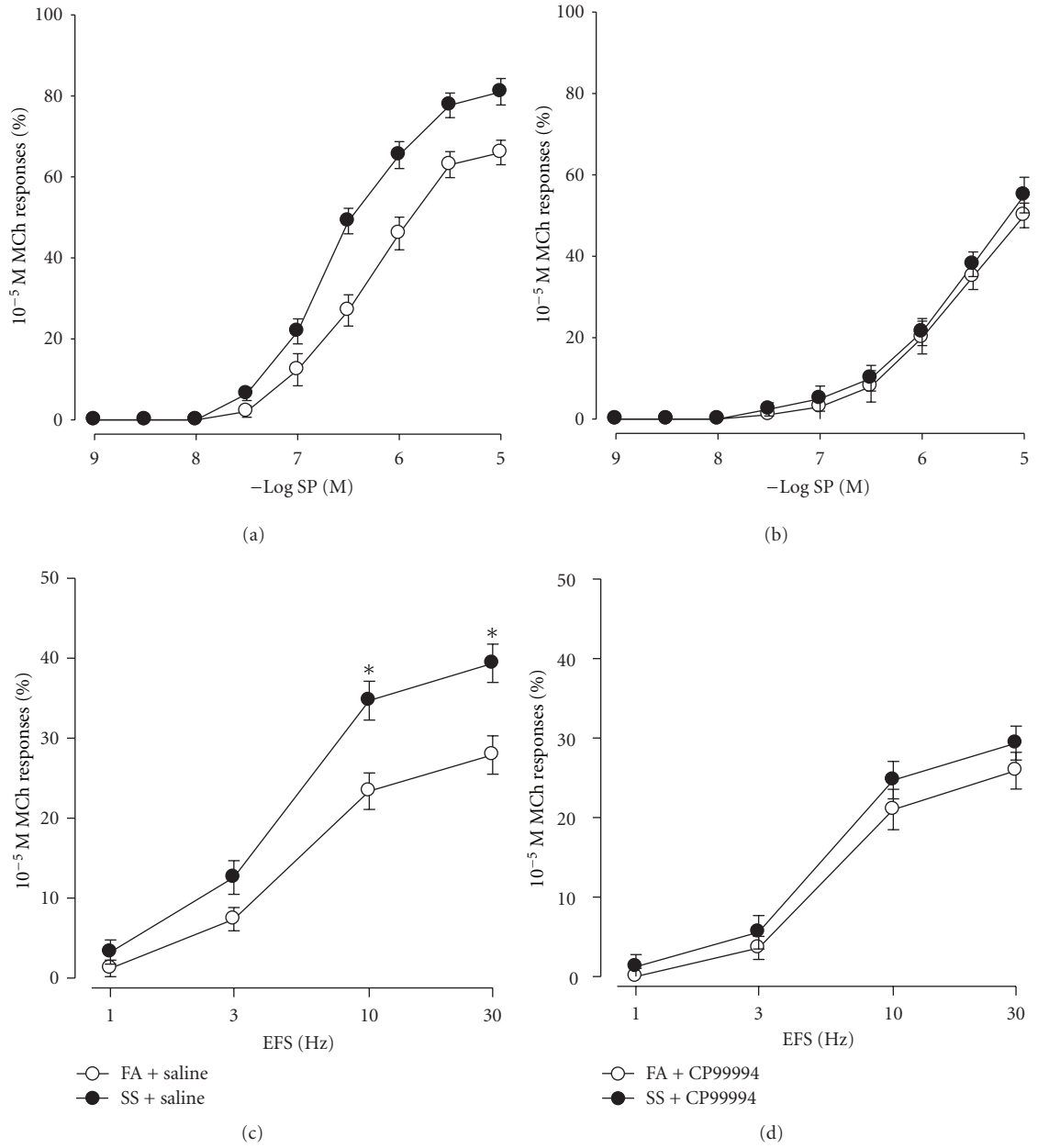

Figure 5: The effect of saline (a and c) or CP-99994 (b and d) on cumulative concentration-response curves for SP and frequency-response curves for EFS in isolated mice tracheal smooth muscle after exposure to 5 days FA (open circles) or SS-(solid circles). Values are means ± SE; $n = 14$ in each group. *Significant difference in EFS between FA and SS exposure, $P \leq 0.05$.

with nonsmoking animals [43]. However, it has also been reported that mainstream cigarette smoking or ETS exposure can enhance allergic airway inflammation [45, 46] and increase airway responses to Ach [45, 47], endothelin [48, 49]. Thus, the relationship between cigarette smoking and airway responsiveness is complex. The different methods and doses of cigarette smoke exposure may induce different airway responsiveness. The low levels of ETS or mainstream cigarette smoke promote allergic sensitization and increase airway responsiveness [46, 50], whereas high levels of mainstream cigarette smoke exposure associated with direct smoking suppress allergic airway inflammation and airway hyperresponsiveness [41–43, 50]. In our present experiment, we used low levels of sidestream tobacco smoke (SS) as a surrogate to ETS instead of mainstream cigarette smoke, and found SS exposure significantly enhances tracheal smooth muscle responsiveness SP and EFS, supporting that low levels of ETS enhance the development of allergic airway inflammation.

Cigarette smoke exposure activates sensory nerve fibers [23–25]. One of the significant findings in the current results from immunohistochemistry found that SS enhances SP expression in airway nerves. These data provide direct evidence that SP nerve fibers in airway smooth muscles were

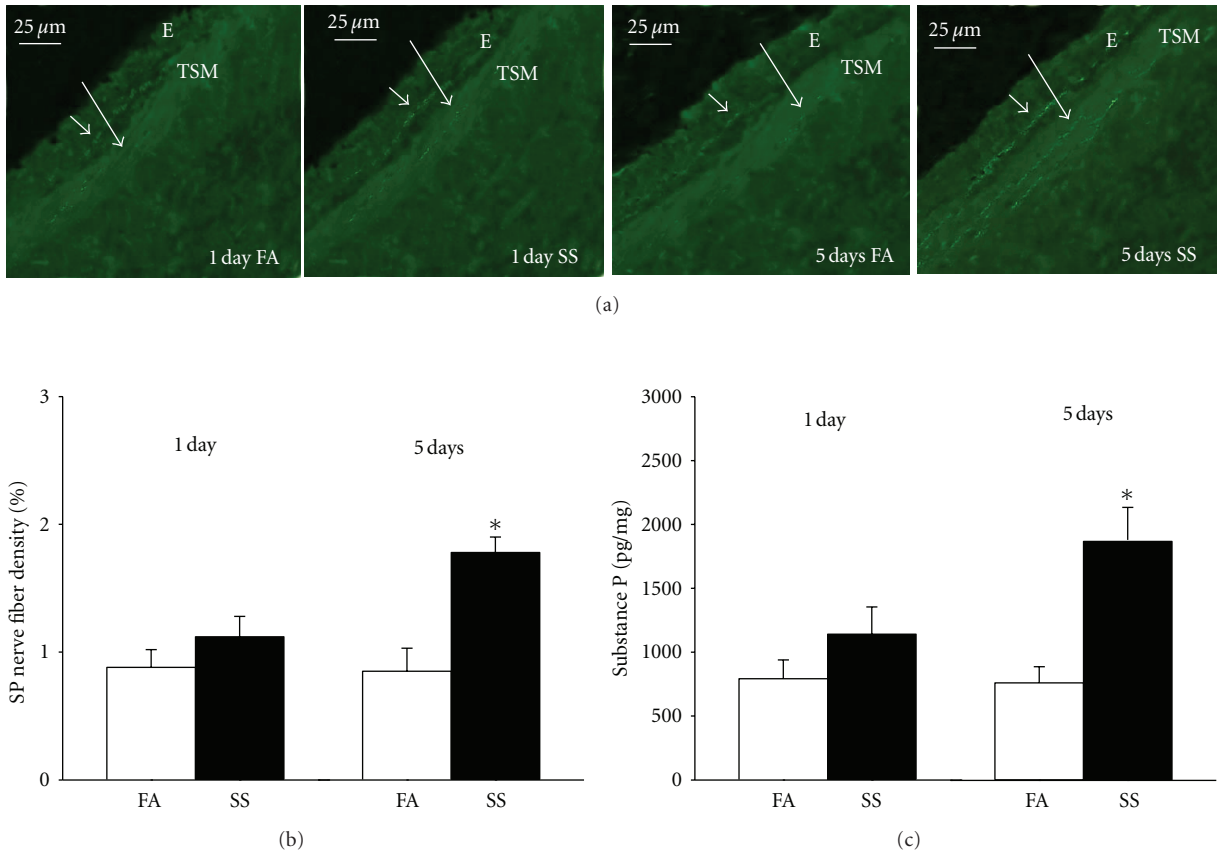

Figure 6: The effect of SS on SP in tracheal smooth muscle and lung. (a) The fluorescence photomicrographs of SP nerve fibers in tracheal epithelium (E) and smooth muscle (TSM) in 1 day and 5 days of FA or SS-exposed mice. Arrows: the localization of SP immunoreactive nerve fibers. (b) The changes of SP nerve fiber density in tracheal smooth muscle after FA (opened bar) or SS (closed bar) exposure. (c) The changes of SP in lung after FA (opened bar) or SS (closed bar) exposure measured by ELISA. Values are means ± SE; $n = 6$ in each group. *Significant difference comparing corresponding data between FA and SS animals, $P \leq 0.05$.

increased by 5 days of SS exposure. Clinic data demonstrated that SP nerve fiber density was increased in airway smooth muscle of severe asthmatics [51]. A more recent study showed that the SP nerve fiber density was increased in airway epithelium from human subjects with persistent nonproductive cough [52]. These studies show that increased levels of SP in human airway sensory nerves may contribute to alteration of airway responses.

In conclusion, our results show that 5 days of SS exposure increases SP nerve fibers innervating tracheal smooth muscle. At the same time, smooth muscle responses to EFS and SP are increased in tracheal segments. Administration of CP-99994, an antagonist of the NK1 receptor, attenuates the SS exposure-enhanced tracheal smooth muscle responses to EFS, indicating that the enhanced responses are dependent on SP increase from sensory nerves or change of NK receptors density in the airway smooth muscle. Combining immunohistochemistry finding that SP nerve fibers were increased in tracheal smooth muscle after 5 days of SS exposure, the current study suggests that the increased SP production by SS exposure may contribute SS-enhanced smooth muscle responsiveness in mice trachea.

Abbreviations

ACh: Acetylcholine
ELISA: Enzyme-linked immunosorbent assay
ETS: Environmental tobacco smoke
FA: Filtered air
MCh: Methacholine
NFD: Nerve fibers density
SP: Substance P
SS: Sidestream tobacco smoke.

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