

Gene expression in asthmatic airway smooth muscle: a mixed bag¹

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Abstract: It has long been known that airway smooth muscle (ASM) contraction contributes significantly to the reversible airflow obstruction that defines asthma. It has also been postulated that phenotypic changes in ASM contribute to the airway hyper-responsiveness (AHR) that is a characteristic feature of asthma. Although there is agreement that the mass of ASM surrounding the airways is significantly increased in asthmatic compared with non-asthmatic airways, it is still uncertain whether there are quantitative or qualitative changes in the level of expression of the genes and proteins involved in the canonical contractile pathway in ASM that could account for AHR. This review will summarize past attempts at quantifying gene expression changes in the ASM of asthmatic lungs as well as non-asthmatic ASM cells stimulated with various inflammatory cytokines. The lack of consistent findings in asthmatic samples coupled with the relative concordance of results from stimulated ASM cells suggests that changes to the contractility of ASM tissues in asthma may be dependent on the presence of an inflammatory environment surrounding the ASM layer. Removal of the ASM from this environment could explain why hypercontractility is rarely seen *ex vivo*.

Key words: asthma, airway smooth muscle, gene expression, protein expression, cytokines.

Résumé : On sait depuis longtemps que la contraction du muscle lisse respiratoire (MLR) contribue de manière significative à l'obstruction réversible du débit d'air qui définit l'asthme. Il a aussi été postulé que des changements phénotypiques dans le MLR contribue à l'hyperréactivité bronchique (HRB) qui caractérise l'asthme. Même si l'on s'accorde sur le fait que la masse de MLR qui entoure les voies respiratoires est significativement plus importante en situation d'asthme comparativement à l'absence d'asthme, on ne sait pas encore avec certitude si des changements quantitatifs ou qualitatifs du niveau d'expression de gènes et de protéines impliqués dans les voies standards de régulation de la contraction chez le MLR pourraient expliquer l'HRB. Cet article de revue résumera les tentatives passées de quantification de changements d'expression génique dans des cellules de MLR de poumons asthmatiques et non asthmatiques, stimulées à l'aide de différentes cytokines inflammatoires. L'absence de données cohérentes issues d'échantillons asthmatiques, couplée à la concordance relative des résultats obtenus à partir de cellules de MLR stimulées suggère que les changements de contractilité du MLR dans l'asthme peuvent être dépendants de la présence d'un environnement inflammatoire autour de la couche de MLR. Le fait de retirer le MLR de son environnement pourrait expliquer pourquoi l'hyper-contraction est rarement observée *ex vivo*. [Traduit par la Rédaction]

Mots-clés : asthme, muscle lisse respiratoire, expression génique, expression protéique, cytokines.

Introduction

Asthma is a disease that affects nearly 300 million people worldwide (Masoli et al. 2004) and is characterized by periods of breathlessness and wheezing caused by narrowing of the airways in the lung. For over 300 years it has been hypothesized that airway smooth muscle (ASM) plays a pivotal role in the development of the symptoms associated with asthma and airway hyper-responsiveness (AHR). In 1698 Sir John Floyer said "I have assign'd the immediate cause of the asthma, to the straitness, compression, or constriction of the bronchia..." (Floyer 1698). However, to date there has been no consensus on whether phenotypic differences in the ASM of asthmatics contributes to AHR (Gunst et al. 2012; Paré and Mitzner 2012). While there is general agreement that there is a greater amount of ASM in mild/moderate, and especially in severe, asthma (Dunnill et al. 1969; Ebina et al. 1993;

Woodruff et al. 2004; Chin et al. 2012; James et al. 2012; Elliot et al. 2014), it is unclear whether the hyperplastic/hypertrophied ASM behaves differently due to altered expression of genes for contractile, cytoskeletal, or inflammatory function.

Increased force production by ASM would be the obvious phenotypic change that could explain the excessive airway narrowing in asthmatics. However, the majority of studies have shown that, when normalized to the amount of ASM present, asthmatic ASM does not produce more force than non-asthmatic ASM (Bai 1991; Cerrina et al. 1989; Chin et al. 2012). When the variable analyzed is shortening capacity or shortening velocity, the results are more heterogeneous. Using human ASM tissues (trachea or bronchi) it was shown that there was no difference in either shortening capacity or velocity of shortening between asthmatic and controls (Bai 1991; Chin et al. 2012). However, using ASM cells from asthmatics, shortening velocity

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Fig. 1. Canonical signal pathways in airway smooth muscle contraction. The green arrow indicates an activation step. The red stop indicates an inhibitory step. The green arrowhead indicates upregulation of gene or protein expression induced by a listed stimulus. Briefly, airway smooth muscle (ASM) is stimulated by agonists binding to their respective G-protein coupled receptors on the surface of the cell, histamine to H_1 receptor, and acetylcholine to M_3 receptor. Activation of the G-proteins leads to activation of 3 pathways. The first, activated through the $G_{\alpha_{q/11}}$ protein, leads to the generation of inositol trisphosphate (IP_3) from membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC). IP_3 liberates calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR) allowing the Ca^{2+} to bind to calmodulin (CAM) to activate myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chain 20 (MLC_{20}), which is part of myosin, to activate cross-bridge cycling and subsequent muscle shortening or force development. In parallel with this pathway, cyclic ADP ribose hydrolase (CD38) can produce the second messenger cyclic ADP ribose (cADPR) to liberate Ca^{2+} from the sarcoplasmic reticulum and eventually lead to muscle contraction. Activation of the $G_{\alpha_{i13}}$ protein activates Rho kinase to inhibit myosin light chain phosphatase (MLCP) and lead to what is known as calcium sensitization by preventing MLCP from dephosphorylating the MLC_{20} . The G-protein G_{α_s} is the protein that activates the relaxing pathway in airway smooth muscle. G_{α_s} activation leads to cyclic AMP (cAMP) production from adenylate cyclase (AC). cAMP activates MLCP to initiate dephosphorylation of MLC_{20} and cross-bridge detachment and eventual muscle relaxation. There are numerous inflammatory mediators or cytokines shown in the figure that can upregulate the expression of a number of these proteins potentially altering the signaling pathways they are a part of. Briefly, interleukin-13 (IL-13) has been shown to upregulate the expression of the H_1 receptor, myosin heavy chain (MYH11), and CD38. CD38 expression has also been shown to be upregulated by interferon gamma ($IFN-\gamma$), tumor necrosis factor alpha ($TNF-\alpha$), and interleukin 1-beta (IL-1 β). There is also evidence that transforming growth factor beta ($TGF-\beta$) upregulates the expression of alpha smooth muscle actin (α SMA) and that immunoglobulin E (IgE) upregulates the expression of MLCK.

and shortening capacity were shown to be increased (Ma et al. 2002; Matsumoto et al. 2007). It is intuitive that if ASM can shorten more, it could narrow the airways more extensively, but it is less clear how increased shortening velocity would affect airway narrowing. It has been shown that increased ASM shortening velocity in rats can account for AHR by allowing more shortening to occur between simulated breaths in an oscillation protocol (Solway and Fredberg 1997; Bullimore et al. 2011).

Another potential difference between asthmatic and control ASM that could contribute to AHR is the muscle's response to the strains imposed on it by tidal breathing and deep inspirations (DIs). A consistent difference between asthmatics and non-asthmatics *in vivo* is the airways' response to strain. DIs protect non-asthmatics from airway narrowing caused by subsequent administration of spasmogen and dilate narrowed airways when DIs are taken after challenge with a spasmogen. These phenomenon are known as bronchoprotection and bronchodilation, respectively, and both are absent or markedly attenuated in asthmatics (Fish et al. 1977; Lim et al. 1987, 1989; Malmberg et al. 1993; Scichilone et al. 2000; Skloot and Toghias 2003). Chin et al. showed that asthmatic tracheal ASM was less responsive to length oscillations mimicking bronchoprotective DIs than tissue from non-asthmatics (Chin et al. 2012).

A different approach to assess whether there are phenotypic differences between asthmatic and non-asthmatic ASM is to examine the tissue's gene expression profile at the mRNA or protein level. Changes to the mechanical properties of the asthmatic ASM that could contribute to AHR would likely be manifested by changes in the expression of the genes or proteins associated with the contractile pathway (Fig. 1). In this report we review the literature concerning the changes in ASM mRNA and (or) protein with a focus on the genes that could alter the contractile function of the muscle. Genetically determined or acquired changes in the expression of myosin light chain kinase (MLCK), myosin heavy chain (MYH11), α -smooth muscle actin (α -SMA), transgelin (TAGLN or SM22), as well as other key proteins involved in contraction (CD38, H_1 receptor) could contribute to changes in ASM force production, shortening capacity or shortening velocity and serve as viable targets to treat the exaggerated airway narrowing that characterizes AHR.

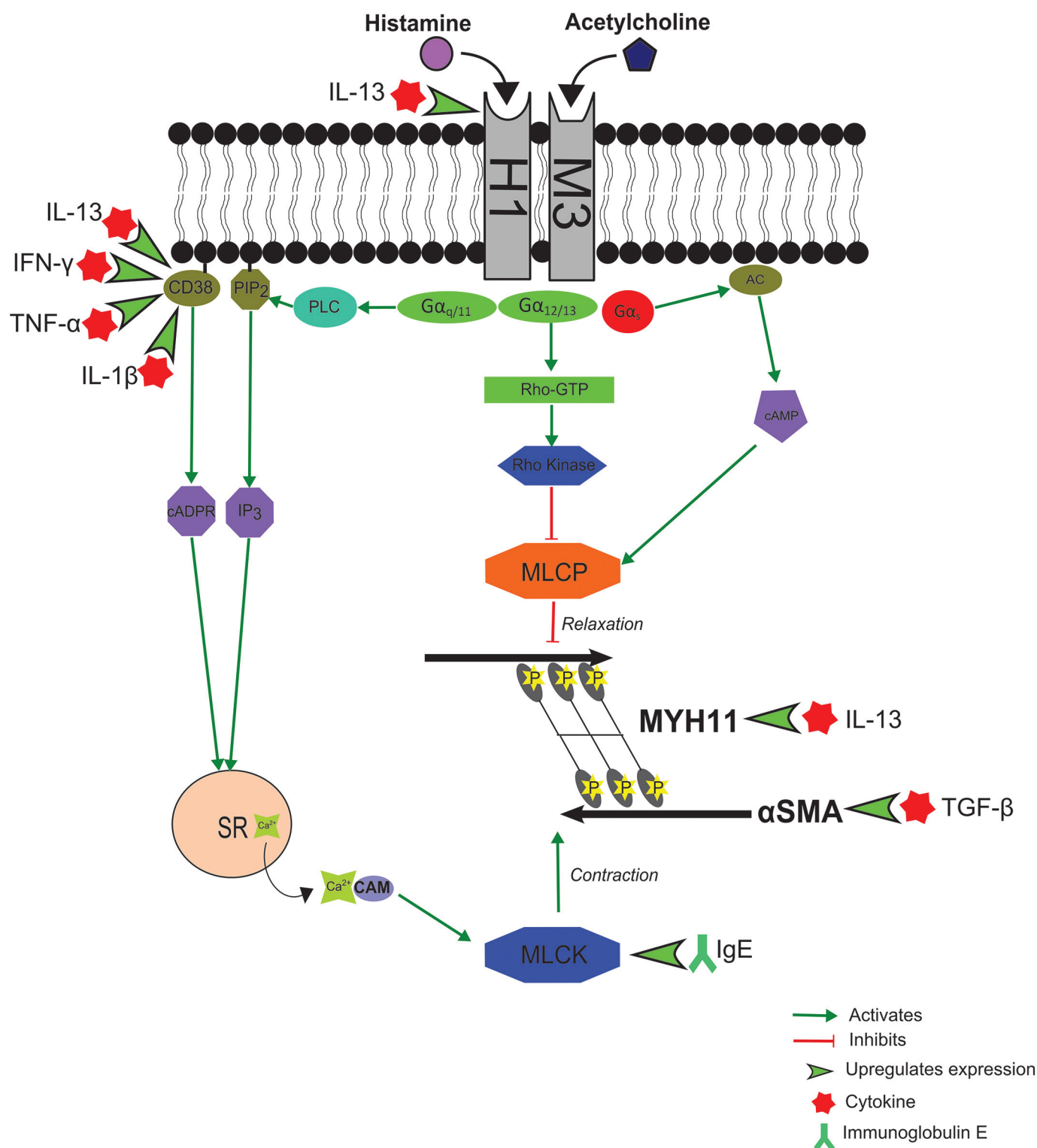
That there is no convincing evidence that asthmatic ASM is phenotypically different from non-asthmatic ASM could be due to the removal of many of the inflammatory mediators in *ex vivo* or *in vitro* experiments. Alternatively there may be no changes in the canonical contractile pathways in asthmatic ASM but rather changes in the expression of genes related to ASM stiffness and response to strain.

Major contractile proteins

Myosin light chain kinase

MLCK is important in regulating contraction in the ASM by phosphorylating the regulatory light chain of myosin (MLC_{20}) upon calcium influx into the cytoplasm (Mabuchi et al. 2010). As such, it has a special role in initiating the force, shortening, and velocity of shortening in ASM and therefore airway narrowing in asthmatics (Zhang et al. 2010). MLCK comes in 4 isoforms, 3 of which are called non-muscle MLCK (nmMLCK), and the last which is called smooth muscle MLCK (smMLCK) (Gallagher et al. 1991). Because of smMLCK's importance in initiating contraction of ASM tissues, it is an attractive target to help explain AHR in asthmatics.

There have been a number of studies looking at the expression of MLCK in samples from asthmatics and non-asthmatics at both the mRNA and the protein level; however, similar to studies analyzing ASM mechanics, there has been no consensus on whether MLCK levels are changed in asthma. In 2002, Ma et al. isolated single ASM cells from bronchial biopsies of mild asthmatic and non-asthmatic subjects to compare their contractility, and used whole biopsies to compare the message level of smMLCK using real-time PCR (Ma et al. 2002). They found that there was a 2.5-fold increase in smMLCK mRNA in asthmatic subjects that was related to a greater shortening capacity in the asthmatic cells. This study was followed up in 2003 by Benayoun et al., who showed that regardless of the type of asthma (intermittent, mild, moderate, or severe) there was an increase in the smMLCK protein expression as assessed by immunohistochemistry (IHC) (Benayoun et al. 2003). Interestingly, there was a further increase in the levels of smMLCK in severe asthmatics compared with those with milder disease. Leguillet et al. showed increased expression of smMLCK mRNA by quantitative PCR in biopsies from mild to moderate asthmatics (Léguillet et al. 2009). The expression of smMLCK protein in the ASM was verified by IHC but not quantified. Increased smMLCK at the gene or protein level is not a universal finding however. The results of 2 studies showed that in bronchial biopsies (Woodruff et al. 2004) and in ASM cells (Matsumoto et al. 2007) there was no difference in either the mRNA or protein levels of MLCK in asthmatics. This was despite the fact that the asthmatic cells studied by Matsumoto et al. produced more extensive contraction in a collagen gel, although the contraction was not faster (Matsumoto et al. 2007). Care should be taken when interpreting the results of gel contraction assays as they are likely to represent contributions from cross-bridge cycling, cytoskeletal remodeling, and remodeling of the collagen gel itself (Ceresa et al. 2009; Wright et al. 2013) and so may not give a true indication of the shortening capacity of ASM. The differences between the results in these 5 studies could be related to numerous things including asthma severity, RNA



isolation or quantification techniques, or removal of ASM tissues from inflammatory environments. In another study, Ammit et al. examined the expression of smMLCK in non-asthmatics who were classified as either sensitized or non-sensitized to allergens (Ammit et al. 2000). The sensitized non-asthmatics displayed greater smMLCK protein expression than the non-sensitized non-asthmatics, possibly indicating that a change in smMLCK may be related to allergic status rather than inflammation. It is interesting to note that the freshly isolated ASM cells expressed only

smMLCK, whereas the ASM cells in culture sometimes expressed nmMLCK in addition to the smMLCK indicating that there may be some phenotype modulation occurring due to the culturing process (Ammit et al. 2000). It is unclear from the results whether the cultured cells express a mixture of the 2 isoforms of MLCK or whether there are distinct populations of cells in culture that express one isoform over the other.

Changes in the expression of several genes and proteins, including smMLCK, have been reported following the stimulation of

cultured non-asthmatic cells with inflammatory cytokines and other related mediators (Balhara et al. 2014). Balhara et al. have recently shown that the gene and protein expression of smMLCK is upregulated in human ASM cells by the allergic antibody immunoglobulin E (IgE) (Balhara et al. 2014). IgE is well known to play a key role in mediating the allergic response of asthmatics, and serum IgE levels in asthmatics are consistently elevated compared with non-asthmatic populations (Borish et al. 2005). IgE is an immunoglobulin produced in B cells that binds to the high affinity IgE receptor (FcεRI) on mast cells or basophils. Interaction of the receptor with IgE-bound allergens causes cross-linking of the receptor and the subsequent release of various inflammatory mediators that can cause smooth muscle contraction (e.g., histamine) or lead to allergic inflammation (cytokines, chemokines and enzymes) (Holt et al. 1999). It has been shown that the high- and low-affinity IgE receptors (FcεRI and FcεRII/CD23, respectively) are expressed in human ASM (Balhara et al. 2014). Balhara et al. demonstrated that IgE increases smMLCK mRNA and protein in human ASM via FcεRI. smMLCK mRNA levels were increased significantly at 6 (~1.5-fold), 24 (~2.2-fold), and 48 (~1.5-fold) hours after the addition of IgE to the cultured cells (but not at 12 h). Likewise, Western blots showed smMLCK protein increased 48 h (~2.5 fold-change) post IgE stimulation (Balhara et al. 2014). Their results suggest that IgE upregulates smMLCK protein via the Syk, ERK1/2, P38, JNK, and PI3K signaling pathways, and that there is a plausible role for IgE to alter the contractile machinery in human ASM. It is possible that smMLCK protein expression may only be increased following stimulation of ASM cells or tissue with IgE, allergens, or inflammatory mediators. If this was true, the results of Balhara et al. would agree with those of Ammit et al. and may explain why in some cases, muscle tissue from asthmatic subjects (removed from the inflammatory environment) display no difference in smMLCK protein or shortening kinetics.

Myosin heavy chain

MYH11 is the major contractile protein in smooth muscle and is composed of a phosphorylatable neck region and a head that can bind to actin to actively shorten the muscle (Sellers et al. 1985). The gene can be alternatively spliced to give rise to 4 distinct isoforms (Eddinger and Murphy 1988). The SM1 and SM2 isoforms differ at the carboxy terminus of the protein with SM1 having a unique 43 amino acid terminus and SM2 having a shorter terminus of 9 unique amino acids (Eddinger and Murphy 1988). The SM1 isoform has been shown to contain a non-helical tail that may affect the shortening velocity of smooth muscle (Hewett et al. 1993; Cai et al. 1995). More recently, studies have suggested that the SM1 and SM2 isoforms control cell length rather than velocity of shortening (Meer and Eddinger 1997). These 2 isoforms are mutually exclusive, meaning that SM1 and SM2 cannot exist within the same protein. The SMA and SMB isoforms arise owing to alternate splicing between exons 5 and 6 (Kelley et al. 1993; White et al. 1993) and are also mutually exclusive. The SMB isoform contains a short 7 amino acid insert between these 2 exons and it has been shown to propel actin faster. This has led to the hypothesis that increased expression of this isoform will result in muscle that shortens faster (Lauzon et al. 1998).

Isoforms of MYH11, including SMA, SMB, and SM2 were shown to be upregulated at the gene level in asthmatics when compared with non-asthmatics (Léguillette et al. 2009) and were negatively correlated with PC₂₀ values; however, these findings did not agree with the results of previous studies (Ammit et al. 2000; Ma et al. 2002; Woodruff et al. 2004). Ma et al. did not detect SMB in their samples, only SMA (Ma et al. 2002). The finding that SMB is present in the ASM and is increased in asthmatic subjects needs to be replicated.

As with MLCK, there is the possibility that the in vivo inflammatory milieu stimulates ASM cells to increase MYH11 expression. Lee et al. examined the expression of ~6500 genes in airway epi-

thelial cells, ASM cells, and lung fibroblasts in response to stimulation with interleukin (IL)-13 (Lee et al. 2001) using a microarray approach. IL-13 is a cytokine produced by Th-2 lymphocytes during allergic hypersensitivity and is a central mediator of asthma (Lee et al. 2001). In ASM cells, IL-13 caused a 3.61-fold increase in MYH11 gene expression over the baseline, suggesting that IL-13 can regulate MYH11 expression in these cells. The work by Lee et al. suggests that IL-13 alone can cause different patterns of gene expression in the 3 airway cell types, including smooth muscle, via a common pathway that involves the transcription factor STAT6. Thus IL-13 produced during allergic inflammation could enhance the contractile response of ASM and contribute to the airway hyper-responsiveness associated with asthma.

Actin

The other major constituent of the ASM contractile apparatus is alpha smooth muscle actin (α-SMA), a protein that forms the filament lattice to which the phosphorylated myosin heads attach to shorten the muscle (Webb 2003). This protein is often used as a marker for mature, contractile smooth muscle cells (Skalli et al. 1986) but is also known to play a role in fibroblast contractility (Hinz et al. 2001). It has been postulated that increased expression of α-SMA or cytoskeletal actins may contribute to the lack of effect of deep inspiration seen in asthmatics (Scichilone et al. 2000) by increasing stiffness and making the muscle more resistant to the force reduction caused by oscillating strain (Wang et al. 2000; Chin et al. 2012). However, to date no difference in the expression of α-SMA in ASM from asthmatics and non-asthmatics has been reported (Woodruff et al. 2004; Léguillette et al. 2009).

Woodman et al. have shown that human ASM cells increase their expression of α-SMA when co-cultured with mast cells or when mast cell-derived β-tryptase was added (Woodman et al. 2008). Tryptases are the most abundant serine-proteinase found in the secretory granules of mast cells, with β-tryptase being the major isoform expressed. β-tryptases have been implicated in the pathogenesis of allergic and inflammatory diseases such as asthma. Woodman et al. also found that ASM-derived TGF-β1 secretion and agonist-provoked contraction were increased in the presence of β-tryptase (Woodman et al. 2008). Overall, it has been suggested that β-tryptase increases TGF-β1 secretion in ASM, which then upregulates contractile protein expression in ASM via autocrine stimulation.

Other proteins

There are numerous proteins other than those described above that are highly expressed in ASM. These include proteins that contribute to solidifying the actin filament network, signaling calcium release, or acting as receptors for spasmogens.

Transgelin/SM22

Transgelin (TAGLN or SM22) is a protein that binds to actin filaments and causes their gelation, leading to a more ordered actin filament network (Shapland et al. 1993). There is little knowledge about the function of TAGLN in ASM, and whether it is over-expressed in the ASM of asthmatics is debated (Woodruff et al. 2004; Léguillette et al. 2009). While Léguillette et al. were able to show an increase in TAGLN mRNA expression in asthmatic airway biopsies, Woodruff et al. did not detect a difference in their sample of asthmatics, although it should be noted that this was a small sample size. The disagreement in the results may be due the use of different tissues, with Woodruff using pure muscle from laser captured tissues and Léguillette using whole biopsies containing ASM, epithelial cells, and fibroblasts. More studies need to be done to determine how changes in TAGLN expression may contribute to AHR in asthma.

CD38

Cyclic ADP ribose (cADPR) hydrolase, or CD38, is a multifunctional cell surface enzyme known to play a role in intracellular Ca^{2+} regulation (Malavasi et al. 2008). Additionally, CD38 catalyzes the synthesis and degradation of cADPR, which regulates Ca^{2+} release in ASM via the ryanodine receptor channel (Deshpande et al. 2003). In asthmatics, increased Ca^{2+} sensitivity could contribute to augmented ASM contraction in response to agonists (see Fig. 1) (Deshpande et al. 2003).

Despande et al. investigated the regulation of CD38 expression and the role of cADPR-mediated calcium release in airway inflammation by exposing cultured human ASM to several inflammatory mediators such as tumor necrosis factor α (TNF- α , 20 ng/mL), IL-1 β (20 ng/mL), or interferon γ (1.0 IU/mL) for 22 h (Deshpande et al. 2003). CD38 mRNA and protein expression increased ~2.1-fold in cells exposed to TNF- α , and there was a similar finding in cells exposed to IL-1 β or INF- γ . Similarly, only TNF- α treatment caused a significant increase in the expression of CD38 protein in the ASM cells. In a follow-up study, Deshpande et al. investigated CD38 expression by cultured ASM cells exposed to IL-13 (50 ng/mL) using a similar experimental protocol (Deshpande et al. 2004). It was found that IL-13-treated ASM cells expressed significantly higher amounts of CD38 mRNA (~2.5-fold increase) and protein (~2.5-fold increase) when compared with the control treatment of bovine serum albumin (Deshpande et al. 2004). In ASM, contraction is highly regulated, and changes in the expression of a calcium-release regulator such as CD38 could affect intracellular calcium levels by modulating its release from the sarcoplasmic reticulum (SR) and thus lead to altered contractile function (see Fig. 1).

Histamine H_1 receptor

Histamine is stored mainly in mast cells and basophils and is a known contributor to allergic diseases such as asthma when levels in tissue or plasma are elevated (White 1990). Through the H_1 receptor expressed on ASM cells and vascular endothelial cells, histamine is able to regulate 2 central mechanisms that contribute to the pathological features of asthma: bronchospasm and mucosal edema (Kaliner 1987; White 1990). When cultured ASM cells were treated with 100 $\mu\text{mol/L}$ histamine, Kotlikoff et al. found that there was a significant increase in intracellular calcium stores (Kotlikoff et al. 1987). This rise in cytosolic calcium is regulated by the H_1 receptor and could indirectly lead to increased airway hyper-responsiveness associated with asthma (Kotlikoff et al. 1987). Jarai et al. investigated the H_1 receptor expression in cultured ASM in response to IL-13 and found that there was a 2.6-fold increase in its gene expression after 4 h (Jarai et al. 2004). More recently, Syed et al. found that the mRNA expression of the H_1 receptor in cultured ASM was increased 1.3-fold in response to stimulation with IL-13 (50 ng/mL) (Syed et al. 2005). Taken together, these studies suggest that IL-13, a central inflammatory cytokine in asthma, can upregulate expression of the histamine H_1 receptor in ASM, likely leading to augmented intracellular calcium release and ASM contractility in response to histamine. However, since AHR is non-specific, mechanisms that affect one agonist pathway, namely histamine-induced contraction, cannot be the sole explanation for AHR.

Proteins unrelated to contractile function

Two recent studies using RNA-seq have identified changes in the expression of genes not directly related to ASM contractile function in asthmatic versus non-asthmatic tissue (Yick et al. 2013, 2014). In their first paper, they micro-dissected ASM from asthmatics and found 174 genes differentially expressed when compared with the controls. Of these genes, 8 could be used to distinguish an asthmatic from a non-asthmatic patient, regardless of their atopy status. These 8 genes are AK8, VANGL1, GPATCH4, RPTOR, HFMI, MTRNR2L8, FAM129A, and LEPREL1, and none have been associated with ASM contractile function. Four of the genes (RPTOR, VANGL1,

FAM129A, and LEPREL1) were associated with AHR. In a second study by this group, they were able to show that asthmatic ASM from patients treated with glucocorticoids had changes in the expression of genes related to cellular growth, proliferation, and development (Yick et al. 2013). Interestingly, prednisone treatment was able to reverse the down-regulation of FAM129A, which was associated with AHR in the first study.

Studies looking into the expression of genes/proteins not normally associated with contractile function in asthmatic ASM might lead to a better understanding of how the ASM is different in asthma. For example, there has been interesting work looking at the expression of actin binding proteins, like zyxin (Rosner et al. 2014), which appears to play a role in stabilizing the cytoskeleton and stiffening ASM cells. Changes in expression of this protein could make asthmatic ASM more resistant to strain and could account for the results seen by Chin et al. (2012). In addition, genome-wide association studies (GWAS) have identified a variant in ADAM33 that is associated with asthma (van Eerdewegh et al. 2002), and work has shown that ADAM33 expression is highly correlated with ASM mechanics in sensitized rats (Lin et al. 2013) and may represent a new target to treat ASM hypercontractility. These results suggest that it might be fruitful to extend analyses of gene expression in asthmatic ASM beyond the "classical contractile" proteins.

Concluding remarks

There is no strong evidence for changes in the expression of contractile proteins MLCK, MYH11 (smMHC), and αSMA in asthmatic ASM as an explanation for AHR and ASM hypercontractility. The lack of definitive results may stem from the variability in airways sampled, sampling techniques, methods used, or may just be an indicator of the heterogeneity of asthma as a disease. Results from studies examining the changes in ASM gene expression in cells stimulated with various inflammatory mediators seem to be more consistent. This indicates that there may not be constitutive changes in the expression of these contractile proteins, but rather they may be induced by the environment in which the ASM cells reside. The concept that it is the milieu of the ASM cells that alters their function was expressed in 2 recent articles (Bossé 2012; Bosse et al. 2012). The majority of studies on the contractile properties of asthmatics have relied on post-mortem samples or cultured cells from asthmatics. As such, samples suffer from gene expression changes associated with steroid treatment (Kelly et al. 2012), removal of samples from inflammatory environments (Bossé 2012), or changes in gene expression associated with obtaining contractile phenotypes in cultured cells (Halayko et al. 1996). Stimulation of non-asthmatic ASM cells with inflammatory mediators may explain how the in-vivo environment of asthmatic ASM cells contributes to their phenotype and potential hypercontractility. A recent study done using rat ASM showed that CD4 $^{+}$ T-cells increase the muscle's shortening velocity, adding support to this theory of inflammation-induced ASM contractile changes (Matusovsky et al. 2014). Future work in this area should focus on replicating the results from cell culture experiments using fully contractile ASM tissue or airway preparations to confirm that what we see is not just an artifact caused by the culturing or serum starvation process. Similarly, the response of asthmatic ASM to inflammatory cytokines and mediators compared with non-asthmatics ASM may prove to be important. It would also be important to show that the inflammatory environment around the ASM indeed contains IgE, interleukins, and other inflammatory mediators. Additional studies on ASM microdissected directly from asthmatic airways (Yick et al. 2013, 2014) may better determine the gene expression changes in asthmatic ASM that occurs in the "asthmatic environment". To date, there still has been no clear consensus on whether the ASM from asthmatic subjects is any more contractile than non-asthmatic subjects.

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