Targeting cytoskeletal biomechanics to modulate airway smooth muscle contraction in asthma

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To contract, to deform, and remodel, the airway smooth muscle cell relies on dynamic changes in the structure of its mechanical force–bearing cytoskeleton. These alternate between a "fluid-like" (relaxed) state characterized by weak contractile protein–protein interactions within the cytoskeletal apparatus and a "solid-like" (contractile) state promoted by strong and highly organized molecular interactions. In this review, we discuss the roles for actin, myosin, factors promoting actin polymerization and depolymerization, adhesome complexes, and cell–cell junctions in these dynamic processes. We describe the relationship between these cytoskeletal factors, extracellular matrix components of bronchial tissue, and mechanical stretch and other changes within the airway wall in the context of the physical mechanisms of cytoskeletal fluidization–resolidification. We also highlight studies that emphasize the distinct processes of cell shortening and force transmission in airway smooth muscle and previously unrecognized roles for actin in cytoskeletal dynamics. Finally, we discuss the implications of these discoveries for understanding and treating airway obstruction in asthma.

Airway smooth muscle (ASM) cells are grouped under the respiratory epithelial layer of the larger airways (trachea and bronchi as well as bronchioles) and less prominently within the lung parenchyma (Fig. 1) (1). Mechanical forces and contractility of ASM tissue are fundamentally responsible for airway narrowing and airway hyper-responsiveness (AHR), which is defined as exaggerated bronchoconstriction in response to contraction-inducing mediators (2). These mechanical forces result from constant variations in lung volume during breathing. ASM tension determines airway caliber throughout respiration and is modulated by breathing (especially deep inspirations) that exert periodic cycles of stretch-recoil on ASM cells (3). Soluble mediators of airway contraction, inflammatory factors, the quantity and composition of extracellular matrix (ECM), and the number and size of individual ASM cells modulate ASM tension. Underlying mechanisms involve both genomic and nongenomic regulation (3). ASM properties change in response to mechanical forces because of adaptive modifications in the organization of the cytoskeleton (4). Several cytoskeleton-related molecules regulate the cytoskeleton and the development of AHR in preclinical cellular and animal models.

The intrinsic mechanophenotype of the ASM cell and its contribution to AHR in asthma is a topic of intense investigation. Apart from specific G protein-coupled receptor (GPCR) antagonists (of muscarinic and leukotriene receptors), which may have clinical efficacy in selected patient populations, ASM-targeted treatments for asthma are primarily focused on increasing relaxation rather than preventing contraction (5). Unfortunately, the therapeutic effects of most relaxation-targeted treatments are acute and transient. Once overcome, the asthmatic airway recontracts. Accordingly, current investigation is focused on the development of drugs with the capacity to prevent ASM cells from hypercontracting in the first place. These treatments primarily target force-generating cytoskeletal pathways in ASM and their relationships to breathing and ECM interactions.

The concept of fluidization–resolidification rests on the premise that the ASM cell is continually in disequilibrium, a state driven by the activity of molecular motors (ATP-dependent fluctuations in protein conformation) and mechanical energy injected into the cell with each periodic stretch. These nonequilibrium dynamics confer three important physical properties to the ASM (1): structural malleability or the ability to switch between a "fluid-like" state that enables cellular migration, division, and deformation and a "solid-like" state that enables structural integrity by generating internal elastic stresses that counterbalance external mechanical forces (6, 7); (2): scale-free rheology, that is, the principle that dynamic cytoskeletal alterations do not occur over fixed relaxation times (8), implying that the ASM cell is close to a solid-like state at rest; (3): universality, that is, the cytoskeletal dynamics obey simple biophysical laws that are applicable over a wide range of molecular interventions, integrative scales (9, 10), and even cell types (11).

We discuss the physiological implications of the ASM fluidization–resolidification framework, describe its molecular origins including dynamically regulated intracellular effectors,

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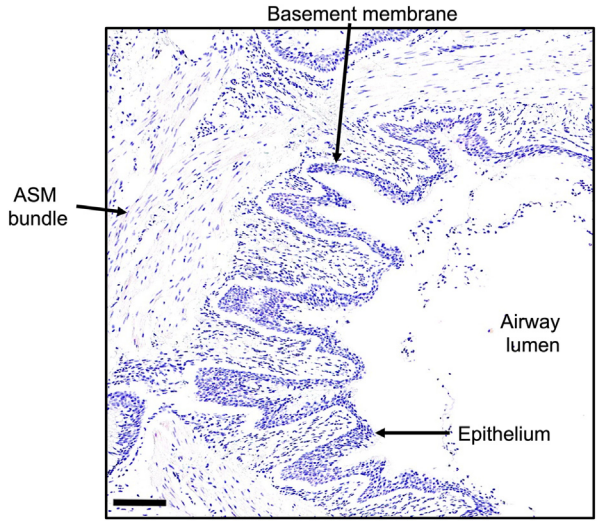

Figure 1. Lung histology. Human lung section stained with hematoxylin shows the airway lumen surrounded by a columnar epithelial layer and basement membrane. Beneath these layers lies the ASM bundle of the bronchiole. Scale bar represents 125 μm. ASM, airway smooth muscle.

mechanotransduction signaling pathways, and the role of mechanical stretch. Throughout, we highlight evidence for dysregulation of these mechanisms in asthma including the influence of asthma-related cytokines. Finally, we describe potential areas of future investigation and emerging therapeutic avenues to modify ASM cytoskeletal biomechanics to combat AHR.

Overview of ASM fluidization–resolidification as a unifying mechanism of contraction

The mechanical behavior of ASM cells bears strong resemblance to that of soft glass materials (12–14). In the absence of external mechanical perturbations, the ASM cell behaves like a solid with a Young's modulus (the ratio of the stress [force per unit area] exerted on the object and the resulting axial strain [displacement or deformation] in the linear elastic region of the material) in the kPa range. However, when subjected to a single transient stretch like that imposed by breathing, the ASM cell promptly and acutely transitions to a fluid-like state. This state is characterized by cytoskeletal remodeling that increases by more than one order of magnitude and cytoskeletal softening and contractile force reduction by greater than 70% (11, 15). The fluidized ASM cell subsequently resolidifies by means of slowed structural rearrangements, cytoskeletal stiffening, and contractile force enhancement (11, 15). Stretch-induced fluidization–resolidification has been observed at the molecular level (6, 16), in the single isolated ASM cell (11), in ASM within intraparenchymal airways of human precision cut lung slices (PCLS) (17) and in isolated lung tissue (18). These dynamic changes correlate with changes in lung airway resistance following a deep inspiration (19).

Fluidization–resolidification of ASM can explain the long-standing observation of the salutary effects of a deep inspiration on airway caliber (20–22). It also provides a conceptual framework to explain ASM–airway dysfunction in asthma. The ASM cell fluidizes only when the applied strain exceeds a threshold value (11, 15). In the bronchial airway, the strain achieved in response to a breath varies inversely as a function of two physical factors: (i) stiffness of the noncontractile elements of other structural cells (*e.g.*, epithelial cells, fibroblasts) and character of the ECM in the airway wall and (ii) stiffness and contractile force generated by the ASM cell itself.

In asthma, increased magnitude and velocity of ASM contraction (23), increased ASM cell number and mass (24, 25), abnormal airway-parenchymal interactions (26), and increased airway wall thickness because of excessive ECM deposition (27) all limit the actual strain imposed on the ASM cell by respiration (Fig. 2). The ASM cell becomes "frozen" in a stiffened/hypercontractile state (28–31), effectively rendering it resistant to the beneficial effects of deep inspiration until either the contracting stimulus is removed or an ASM relaxant is delivered. In this review, we discuss fluidization—resolidification of the ASM cell and the failure of the ASM to fluidize in asthma as unifying mechanisms that underlie AHR. Advances in our understanding of these concepts have revealed previously unrecognized pathways for therapeutic intervention.

The molecular origins of stretch-induced fluidization–resolidification

Stretch-induced structural rearrangements are rapid. A transient stretch–unstretch maneuver occurs through fast F-actin disassembly and slow F-actin reassembly, with a magnitude and duration that mirrors the time course of fluidization–resolidification (32). Cofilin severs F-actin during

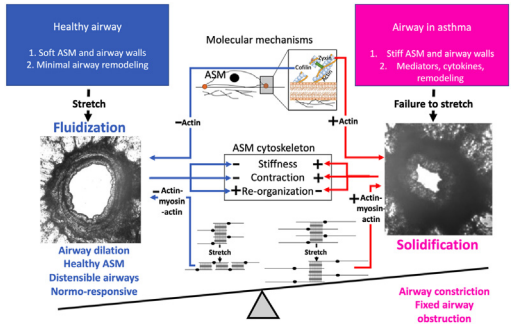

Figure 2. A proximate cause of bronchoconstriction in asthma is failure of the ASM to fluidize. The microenvironment of every cell resident within the airway wall possesses physical as well as humoral attributes. Inhaled corticosteroid (ICS)–insensitive humoral pathways (*red*) dispose toward airway remodeling and persistent asthma, whereas physical pathways (*blue*) are ordinarily sufficient to hold those humoral effects in check. These pathways include positive feedback loops that have potential to incite instability. *Left*, in the healthy airway, the microenvironment is soft and distensible, with minimal remodeling. With each deep breath, the actin in ASM dissociates, partly mediated by the actin severing protein cofilin and disruption of the actin (*thin, black, short filaments*) to myosin (*thick, gray*) connection. This leads to ASM fluidization, a physical state defined by ASM softening, force reduction, and the enhancement of molecular rearrangements. *Right*, in asthma, the airway is stiff, inflamed, contracted, remodeled, and with each deep breath, fails to stretch. The ASM solidifies, a physical state defined by stiffening, contraction, and molecular arrest. Molecular effectors include zyxin and enhanced actin (*thin, black, long filaments*) to myosin (*thick, gray*) connectivity. ASM, airway smooth muscle; FIR, fluctuation induced relengthening; latch, latch state.

stretch-initiated fluidization. Cofilin knockdown in ASM cells blunts fluidization compared with control cells without affecting resolidification (33). Further, cofilin-mediated actin depolymerization is limited to the unstretch phase and is preferentially stimulated by a decrease in tension (34, 35).

Studies of reconstituted actin–myosin networks *in vitro* have revealed that stretch-induced fluidization is mediated by disruption of myosin crosslinks (36) and actin–myosin crosslinks (37, 38). Forcible ASM stretch with short-actin filaments causes relative sliding of the filaments and a loss of the "overlap" zone comprising two filaments connected in tandem to a single myosin filament. The reorganization of actin filaments from a parallel to a "series" arrangement reduces their concerted contractile capacity. However, this mechanism alone cannot account fully for ASM cytoskeletal plasticity (7, 8), scale-free deformability (39), differential response to a homogenous *versus* a heterogeneous stretch (15), or the rapid F-actin disassembly followed by a gradual reassembly. Likewise, disruption of myosin crosslinks alone cannot explain the universality of the fluidization–resolidification response amongst muscle and nonmuscle (NM) cells (11).

The actin filament–restoring protein zyxin, which localizes to focal adhesions (force bearing, protein-rich hubs that connect the ASM cell membrane to the ECM), has a key function in the resolidification phase (40). Zyxin expression increases with acute stress fiber fragmentation, and zyxin knockdown in human ASM (HASM) or mouse PCLS does not affect fluidization but strongly reduces resolidification and associated recovery of force (40). Notably, there is increased accumulation of zyxin in lungs of patients with fatal asthma compared with nondiseased controls.

Strikingly, disruption of signaling cascades previously implicated in cellular responses to a prolonged stretch including PI3 kinase, Akt, Rho-associated coiled-coil forming kinase (ROCK), and mitogen-activated protein kinase kinase pathways has no noticeable effects on the acute fluidization–resolidification response in smooth muscle cells (32). Although transient stretch does not alter overall protein tyrosine phosphorylation patterns (32), generalized tyrosine phosphorylation induced by phenylarsine oxide, a membrane-permeable tyrosine phosphatase inhibitor, blunts the resolidification response (15). However, phenylarsine oxide's effects might be due to decreased cellular ATP content, which is known to impede resolidification (11).

Molecular origins of contractile forces leading to ASM solidification—GPCR-evoked cell shortening

Two principal mechanisms operating independently of one another induce ASM solidification and increased contractile capacity. G protein signaling causes ASM cell shortening through actin and myosin filament crossbridging (41). In parallel, actin filament polymerization and adhesome complex formation increase cytoskeletal rigidity, which in turn exerts tension on cell membranes to transmit force to neighboring ASM cells and the underlying lung tissue *via* ECM proteins.

Among the most well-studied contractile mediators in the airways are acetylcholine (ACh), which is released from efferent vagal nerves, and histamine and leukotrienes, which are derived from lung resident mast cells. ACh, histamine, and leukotrienes and most other contractile agonists bind GPCRs, which activate heterotrimeric G proteins, typically those containing Gαq/11, through the exchange of GDP for GTP (Fig. 3) (42). Gαq–GTP stimulates phospholipase β to generate inositol 1,3,4 triphosphate and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate within the membrane. Inositol 1,3,4 triphosphate activates cognate receptors on the sarcoplasmic reticulum (SR), which releases Ca^{2+} from intracellular stores. Ca^{2+} binds to calmodulin, which in turn elicits the phosphorylation and activation of myosin light chain kinase (MLCK) by Ca^{2+}–calmodulin-dependent kinase. Muscarinic m3 receptors on ASM bind ACh and its analogs carbachol (CCh) and methacholine (MCh) also activate Gα12, which in turn activates several effectors including PI3K and RhoA. PI3K–RhoA-mediated activation of tROCK regulates MLC phosphorylation (43). Regulators of G protein signaling and GPCR-related kinases govern G protein activity. We have recently reviewed the extensive contributions of these two protein families to AHR and asthma elsewhere (44).

Type II myosin, which is made up of two myosin heavy chains that intertwine to form a tail and bind actin at the head and neck, mediates cell shortening. This interaction is regulated by the association and phosphorylation of two associated light chain pairs (MLC17 and MLC20). MLCK phosphorylates MLC20 at Ser19, which promotes the interaction with actin filaments. Repetitive cycles of actin–myosin crossbridging along the cell's longitudinal axis cause shortening and solidification through the development of tension (45).

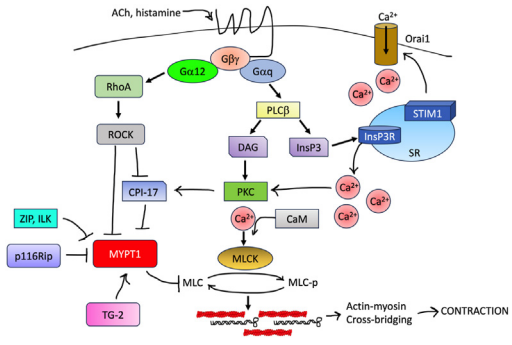

Figure 3. Solidification mediated by force generation pathways in ASM. Contraction-inducing mediators (CCh, histamine) activate GPCRs coupled to Gαq and/or Gα12. G protein activation stimulates activity of phospholipase Cβ (PLCβ), which generates an inositol 1,4,5-trisphosphate (Ins_{P3}) and diacylglycerol (DAG). Ins_{P3} induces release of Ca^{2+} from sarcoplasmic reticulum stores. Cytosolic Ca^{2+} binds calmodulin and activates myosin light chain kinase (MLCK). MLC phosphorylation on Ser19 by MLCK causes crossbridging of myosin filaments (*black*) with actin (*red*) and cell shortening along the longitudinal axis. Extracellular Ca^{2+} enters the cell through membrane-embedded channels (Orai1). In collaboration with the Ca^{2+} sensor STIM1, Orai1 mediates oscillations in intracellular Ca^{2+} levels that are required to sustain contraction. GPCRs also stimulate activity of guanine nucleotide exchange factors (GEFs) for RhoA. Activated RhoA stimulates ROCK-mediated inhibitory phosphorylation of MYPT1, which prevents MLC dephosphorylation and prolongs contraction. Other inhibitors of MYPT1 activity include CPI-17 and p116Rip. ASM, airway smooth muscle; CCh, carbachol; GPCR, G protein-coupled receptor.

Intracellular origins of contractile forces leading to ASM solidification—actin–myosin interactions

Fluctuating intracellular Ca^{2+} concentrations continuously regulate actin–myosin interactions. Sustained excitation–contraction coupling requires the repletion of intracellular Ca^{2+} stores, which occurs primarily through Ca^{2+}-induced Ca^{2+} release (store-operated Ca^{2+} entry) (46). The influx of extracellular Ca^{2+} into the cytosol through plasma membrane (PM)–associated store operated Ca^{2+} channels (Orai1) in collaboration with the SR-associated Ca^{2+}-sensing protein stromal interaction molecule 1 (STIM1) generates repetitive oscillations in intracellular Ca^{2+} levels (Fig. 3). Voltage-dependent L-type Ca^{2+} channels on the PM and ryanodine receptors on the SR also contribute to the maintenance of intracellular Ca^{2+} concentrations and contractility (47). Store-operated Ca^{2+} entry dysfunction may increase susceptibility to AHR. Airways in PCLS from Balb/c mice contract more effectively and have exhibited faster ASM Ca^{2+} oscillations in response to contraction-inducing mediators than those from C57BL/6 mice. These changes correspond with higher expression of STIM1 and Orai1 and increased AHR in asthma models (48). Type 2 cytokines IL-4 and IL-13 also induce Orai1 expression in HASM and *in vivo* in rodent asthma models (Table 1) (49, 50).

An extensive network of intracellular proteins dynamically controls myosin activation and actin crossbridging. Myosin light chain phosphatase, composed of a PP1c catalytic subunit and regulatory subunit (MYPT1), inhibits ASM contraction by binding myosin and dephosphorylating MLCK (51). Phosphorylation of MYPT1 on Ser507, Thr696, and Thr853 by ROCK and other kinases may inhibit myosin light chain phosphatase activity to prolong contraction. ROCK expression is increased in ASM from bronchi and bronchioles of lung tissue from patients with asthma compared with controls (52). Genetic deletion of RhoA or ROCK or the use of pharmacological inhibitors reduce AHR in mouse models of asthma (53, 54). However, mechanistic studies suggest that the RhoA pathway facilitates ASM solidification primarily by promoting actin polymerization rather regulating MYPT1 activity (55). The RhoA effector rhotekin induces NM myosin polymerization at the cell cortex (submembranous region) through an interaction with S100A4 (Fig. 4) (56). NM myosin filaments act as a platform for proteins required for the assembly of F-actin.

Additional mechanisms exist to limit actin–myosin-mediated solidification independent of RhoA. Several kinases (ZIP kinase, integrin-linked kinase [ILK]) inhibit MYPT1 activity directly or indirectly by activating the phosphatase CPI-17 (57). p116(Rip), whose expression in increased in ASM from patients with asthma, interacts with F-actin, myosin, and MYPT1 (58). Transgelin 2, a transmembrane protein activated by endogenous proteases such as metallothionein 2 -attenuates bronchoconstriction *ex vivo* (59). Administration of metallothionein 2 or TG12 agonists reduces AHR in allergen-challenged mice with minimal effects on allergic lung inflammation (Table 2). TG-2 agonists inhibit contractile signaling through several mechanisms including MYPT1 dephosphorylation and inhibitory RhoA and ezrin phosphorylation (60).

Contractile force transmission leading to ASM solidification—adhesome complexes

Membrane–cytoskeletal and membrane–ECM interactions have a pivotal role in ASM solidification. ASM cells transmit force to neighboring cells or the underlying lung tissue upon sensing mechanical strain–stress through integrin receptors. Integrin activation leads to the recruitment of cytoskeletal and signaling proteins at the cytosolic face of the PM in adhesome complexes (1). Adhesomes transmit tension generated by cell shortening to outside the cell membrane while simultaneously transmitting external mechanical forces to the ASM cell interior.

Integrins are single-pass integral cell membrane proteins consisting of alpha and beta subunits, which form various heterodimers that bind to various ECM components, including collagen, laminin, and fibrin through their extracellular domains (Fig. 5*A*) (61). HASM primarily express α1-, α3-, β1-, β3-, and β5 integrins (1). Integrins have different affinities for ECM ligands based on their ability to adopt distinct conformations. Integrin ligand-binding recruits intracellular adhesome complexes of talin, α-actinin, ILK, and focal adhesion kinase (FAK) to its cytosolic domain, a process termed "outside–in" signaling. ACh recruits talin to the integrin cytosolic domain, causing also them to adopt a high affinity "open" conformation—termed "inside–out" signaling (62).

Table 1
ASM cytoskeleton-related proteins dysregulated in asthma

Protein	Function	Comment	References
Zyxin	Actin stabilization	Increased lung expression in fatal asthma	(40)
L-type Ca^{2+} channel (LDVCC)	Ca^{2+} oscillations	Upregulated in ASM by TNFα	(46)
STIM/Orai1	SOCE/Ca^{2+} oscillations	Expression increased by type 2 cytokines	(49, 50)
ROCK	Inhibitory MYPT1 phosphorylation	Increased bronchial expression in asthma; ROCK inhibitors reduce AHR	(52)
RhoA	MLC phosphorylation	ASM expression increased by IL-13	(57)
p116 (Rip)	RhoA-independent inhibition of MLC phosphorylation	Decreased expression in ASM cells in asthma	(58)
Nestin	Promotes actin polymerization	Increased in ASM in asthma	(87)
ARHGEF1	RhoA activation	Upregulated in ASM from subjects with asthma and zyxin allergen challenged mice	(103)
IL-31 receptor	Potentiation of m3 muscarinic receptor mediated contraction	Upregulated by IL-4 plus IL-13	(104)

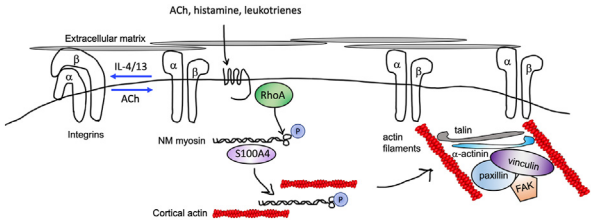

Figure 4. Integrin-mediated mechanosensing initiates cytoskeletal remodeling. ASM cells adhere to extracellular matrix through discrete attachments called focal adhesions (or adhesomes) enriched in proteins that regulate cytoskeletal dynamics. In resting cells, integrins exist in a closed (inactive) dimeric conformation. Contractile mediators (acetylcholine [ACh]) promote adoption of an open, active integrin conformation and RhoA-dependent phosphorylation of type 2 myosin and association with accessory proteins such as S100A4, which facilitates myosin polymerization and crosslinking to cortical (peripheral) actin. Peripheral actin filaments provide a scaffold for integrin linkage to intracellular actin filaments and coupling with adhesome proteins including talin, α-actinin, vinculin, and paxillin. Type 2 cytokines (IL-4/13) prevent talin binding to integrins and formation of the open conformation. ASM, airway smooth muscle.

Airway inflammation and remodeling associated with established asthma (63), including increased deposition of ECM proteins, may alter integrin functions (64). IL-4 or IL-13 promotes formation of a low-affinity "bent" conformation and proteolytic inactivation of talin (65, 66). Disruption of α5β1 or α2β1 integrin binding to collagen I or fibronectin, respectively, inhibits IL-13-induced enhanced contraction of human bronchial rings in response to MCh and reduces airway resistance in allergen-challenged mice (67), indicating that integrin–ECM interactions contribute to AHR pathogenesis and may represent an effective therapeutic target.

Contractile force transmission leading to ASM solidification—focal adhesions and actin polymerization

Integrin activation and assembly of adhesome complexes occur at FAs, where various regulators of actin polymerization and depolymerization assemble and disassemble in a dynamic and highly regulated fashion to mediate solidification or fluidization, respectively.

Talin and α-actinin form heterodimers that bridge actin filaments to activated integrins and facilitate binding of vinculin, paxillin and kinases including ILK and FAK (Fig. 4). Blockade of the talin-α actinin interaction reduces tension development in canine tracheal rings without affecting MLC phosphorylation or actin polymerization (68). ACh induces RhoA- and phosphorylation-dependent conformational changes in vinculin (complexed with paxillin), which exposes binding sites for talin, α-actinin, and actin filaments (69, 70). In turn, FAK dependent phosphorylation of paxillin recruits neuronal Wiskott–Aldrich syndrome protein (N-WASp) indirectly through the SH2 domains of the adapter protein CRKII (70). N-WASp-CRKII complexes bind guanine nucleotide exchange factors (GEFs) for the small GTPase cdc42 including DOCK180 and PIX, which leads to activation of Cdc42 and N-WASp coupling to the Arp2/3 complex (Fig. 5*A*) (4, 71). Arp2/3 are structurally similar to G-actin and provide a foundation for the formation of side branches on pre-existing actin filaments (41). F-actin filament elongation is mediated by vasodilator-stimulated phosphoprotein (VASP), which is anchored to the adhesome by activated vinculin. VASP tetramers catalyze incorporation of G-actin at expanding actin filament tips in coordination with profilin-1 (Pfn-1). ACh stimulation induces VASP phosphorylation on Ser157, which is required for the regulation of ASM actin dynamics and contraction in a vinculin-dependent manner (72).

Mechanotransduction pathways leading to ASM solidification—kinase regulators of adhesome functions

Several non-receptor kinases and related proteins regulate actin remodeling-dependent solidification in adhesomes dynamically. Contractile stimulation recruits the cytosolic IPP (ILK–PINCH–Parvin) complex consisting of ILK, the adapter PINCH (Particularly Interesting New Cysteine-Histidine rich protein), and α-parvin, an actin-binding protein, to integrin-linked adhesion sites to promote solidification (Fig. 5*B*) (73). Although ILK phosphorylates paxillin in other smooth muscle

Table 2
Therapeutic interventions targeting cytoskeletal mechanics in asthma—Implications for fluidization and solidification

Drug/compound	Target	Fluidization–solidification mechanism(s)	Comments	References
Y16	ARHGEF12	Actin polymerization	Attenuated IL-17 induced bronchial ring hypercontraction	(53)
Fasudil/hyroxyfasudil	ROCK	Reduced MYPT1 phosphorylation	Decreased AHR in mouse models and PCLS; anti-inflammatory	(54)
TSG12/TSG1180	Transgelin-2	MYPT1 dephosphorylation; RhoA, ezrin phosphorylation	Elicit bronchodilation and reduce AHR in mouse models	(59, 60)
Volasertib (BI6727)	Plk1	Actin polymerization	Relaxation of mouse bronchial rings; reduced HDM associated AHR; Phase III clinical trials for cancer	(77)
Imatinib	c-Abl	Actin polymerization	Reduced AHR in short term RDBPC trial of patients with severe asthma	(81)
FR900359	Gαq	Ca^{2+} flux, MLC phosphorylation	Decreased AHR in several animal models of asthma	(107)
R59949	diacylglycerol kinase (DGKζ)	Ca^{2+} flux (PLCβ)	Decreased AHR in mouse models and PCLS; anti-inflammatory	(108, 109)
Pitavastatin, atorvastatin	RhoA	Inhibits MLC phosphorylation, actin polymerization	Reduces AHR in mice and PCLS contraction; lower risk of exacerbations in people treated with statins for dyslipidemia	(116, 117)

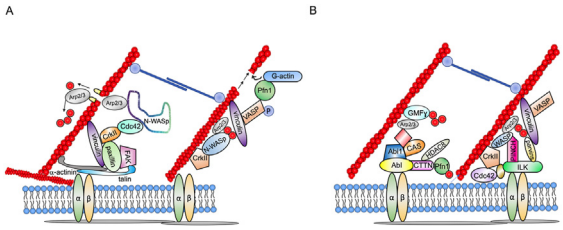

Figure 5. Mechanisms of actin polymerization and regulation. *A*, phosphorylation of paxillin by focal adhesion kinase (FAK) recruits N-WASp to the adhesome complex through interactions with the adapter protein CrkII. Paxillin phosphorylation recruits GEFs for cdc42 (G protein receptor kinase interacting tyrosine phosphorylated [GIT and Pak-interacting exchange factor (PIX)]) to the complex. WASp binds to activated (GTP-bound) cdc42 and activates Arp2/3, a protein complex structurally related to G-actin that binds existing actin filaments and promotes branching. Tetramers of VASP bind vinculin and elicit actin filament elongation by recruiting profilin (Pfn-1) bound to G-actin. *B*, actin polymerization–depolymerization is regulated by kinases including the IPP (integrin-linked kinase [ILK], particularly interesting new cysteine–histidine-rich protein [PINCH], parvin) complex, c-Abl (linked to the adhesome by the adapters CAS and Abi1), and by nonkinase regulators including glia maturation factor γ (GMFγ), histone deacetylase 8 (HDAC8), and cortactin (CTTN).

types, the specific substrates of ILK in ASM have not been firmly established (74). p21-activated kinases (PAKs) constitute a family of ROCK activated Ser/Thr kinases that elicit actin polymerization by means of paxillin phosphorylation on Ser273, recruitment of cdc42 GEFs GIT1 and βPIX, and cdc42 activation (55).

The PAK-related kinase, Ste20-like kinase (SLK) also promotes actin-dependent solidification by means of paxillin phosphorylation on Ser272 by Polo-like kinase (Plk), which elicits N-WASp activation (75, 76). Plk1 antagonism shows therapeutic promise; a specific inhibitor (Volasertib, BI6727) impairs bronchial ring contraction and the development of AHR induced by house dust mite (HDM) sensitization and challenge in mice (77). Moreover, treatment of MCh-precontracted mouse tracheal rings with volasertib induces relaxation, suggesting its application as a bronchodilator. Plk1 and PAK1 may also have a role in fluidization through phosphorylation of the intermediate filament protein vimentin, which leads to disassembly of the WASp-Arp2/3 complex (73, 77).

The Src family kinase c-Abl regulates solidification through interactions with Abelson interactor 1 (Abi1) and CAS, which is essential for N-WASp-mediated F-actin formation in ASM (73). Expression of both c-Abl and Abi1 is increased in mouse asthma models and ASM cells from patients with severe asthma (78, 79). Smooth muscle-specific c-Abl knockout or administration of c-Abl inhibitors imatinib or GNF-5 reduces bronchoconstriction in models of chronic asthma in mice. Moreover, Imatinib and GNF5 exert additive effects on β-agonist-evoked bronchodilation, suggesting that they also affect fluidization (80). Notably, in a short term, randomized, double blind, placebo-controlled study of patients with severe asthma, imatinib reduced AHR and lung mast cell counts over a 24-week period (81).

Mechanotransduction pathways of ASM solidification—inducers of actin filament polymerization

Actin polymerization is a complex, dynamically coordinated process. Profilin-1 (Pfn-1) catalyzes the exchange of ADP for ATP on monomeric G-actin, releasing actin monomers from thymosin-β4, an actin sequestration protein (82) for incorporation into the barbed (fast growing) ends of actin filaments through interactions with VASP. Pfn-1 also promotes solidification by preventing spontaneous Arp2/3-mediated actin filament branching (83). Pfn-1 interactions with cortactin are also critical for solidification. Treatment with a cell permeable decoy peptide (CTTN-1) inhibits actin polymerization in HASM and contractile force development by tracheal rings by inhibiting cortactin-Pfn-1 interactions and F-actin formation independent of MLC phosphorylation (82). Cortactin is highly regulated by HDAC8-mediated deacetylation (84) and c-abl mediated phosphorylation on Tyr412, and interaction with the adapter protein CAS (85). Human genome-wide association studies (GWASs) have uncovered an intronic SNP in *CTTN* (rs3802780) that confers increased risk of severe asthma (odds ratio: 1.71).

Nestin is a type VI intermediate filament protein that mediates solidification (86). ACh elicits Plk1-mediated nestin phosphorylation on Thr315, which in turn leads to the formation of a nestin–Plk1–vimentin complex and actin polymerization through the recruitment of Pfn-1 and cortactin to the PM. Nestin expression is increased in ASM cells from subjects with asthma compared with controls. Smooth muscle–specific nestin knockout in mice reduces AHR in a model of chronic asthma induced by long-term HDM challenge in part because of reduced ASM hyperplasia and Th2 inflammation (87).

Mechanotransduction pathways of ASM fluidization—regulators of actin depolymerization/filament stability

Cofilin, implicated in fluidization elicited by ASM stretch, is a member of a family of actin-depolymerization factor proteins that severe F-actin when dephosphorylated on Ser-3 by calcineurin in a Ca^{2+}-dependent fashion. Cofilin also mediates actin filament debranching by removing the Arp2/3 complex (88). Cofilin activation is required for ACh-induced tension development and maintenance of tone in tracheal smooth muscle (89, 90). Coronins constitute another family of proteins that bind F-actin and Arp2/3 in yeast and can promote actin oligomerization but have not yet been studied in ASM (88). GMFγ (glia maturation factor γ) is an actin debranching factor that is highly expressed in ASM and localizes to FAs (91). At homeostasis, GMFγ binds tonically to the Arp2/3 complex, eliciting F-actin disassembly and inhibiting nucleation of new actin filaments. ACh stimulation of HASM induces c-abl-mediated GMFγ phosphorylation and dissociation from the Arp2/3 complex. GMFγ knockdown by siRNA reduces contraction of human bronchial rings *ex vivo* and actin polymerization in HASM (92).

β-catenin forms a complex with membrane-associated cadherins (N-cadherin in ASM) and regulates F-actin filament stability through actin-binding proteins including α-catenin, α-actinin, and p120 catenin (93). Knockdown of β-catenin or a pharmacological inhibitor of β-catenin–N-cadherin interactions inhibits ASM contraction through

unclear mechanisms that are independent of actin polymerization or MLC phosphorylation (94, 95).

Effects of asthma-related cytokines on ASM solidification

Several type 2 cytokines increase the severity of AHR by augmenting solidification pathways. IL-4 and IL-13 enhance contraction of human bronchial rings to histamine and leukotriene D4 by upregulating expression of mRNA cognate receptors (*HRH1* and *CYSLTR1*, respectively) (96). Accordingly, dupilumab, a therapeutic antibody antagonist of IL-4Rα, blocks this response (96). IL-13 also increases the expression of RhoA and CPI-17 in human and mouse ASM (57), suggesting that it also promotes solidification by regulating actin–myosin interactions and actin polymerization.

Other asthma-related cytokines may alter solidification mechanisms. IL-17, which has been implicated in the pathogenesis of severe, steroid-resistant asthma, enhances RhoA activation in ASM by means of the RhoGEF ARHGEF12 (97, 98). Treatment of mice with a RhoGEF inhibitor (Y16) weakens IL-17A-induced tracheal ring hypercontraction, and *Arhgef12*−/− mice have decreased allergen-induced AHR compared with controls (53). Tumor necrosis factor alpha, a cytokine frequently elevated in the airways of patients with type 2 low asthma (99), upregulates voltage-dependent L-type Ca^{2+} channel expression in mouse ASM (47). Other studies suggest that tumor necrosis factor alpha increases force generation in porcine tracheal smooth muscle by upregulating actin expression and polymerization with little effect on MLC phosphorylation (100). Transforming growth factor beta (TGFβ) is upregulated in airways of patients with established asthma and contributes to remodeling (101). TGFβ pretreatment of HASM enhances CCh-induced Ca^{2+} flux, MLC and MYTP1 phosphorylation in an ROCK-dependent but RhoA-independent fashion (102). A separate study found increased expression of the RhoGEF ARHGEF1 in ASM cells from subjects with asthma, TGFβ-treated ASM cells from healthy donors, and allergen-challenged mice. ARHGEF1 knockdown suppresses TGFβ-induced hypercontraction of bradykinin-stimulated rat bronchioles (103). The IL-31 receptor (IL-31R) is expressed on human and mouse ASM cells and is upregulated by IL-4/13 and in allergen-challenged mice. While IL-31 itself has no direct impact on contractility, IL-31R forms a complex with m3 muscarinic receptors to stabilize m3R expression. IL-31R binds m3R and enhances agonist-induced Ca^{2+} flux and MLC phosphorylation in ASM (104).

Cytokine-induced changes in ASM contractile responses can inform GWAS of asthma. Specifically, the results of ASM contraction measurements in cells from the same donors used for transcriptomic and epigenetic studies revealed three significant outcomes: (1) IL-13 and IL-17A altered expression of ASM genes that were among the most prevalent variants in asthma GWAS studies; (2) IL-17A-induced changes in contractility were highly correlated with changes to the transcriptional landscape; (3) molecular quantitative trail loci for ASM gene expression and cellular quantitative trait loci for ASM contraction were enriched in asthma GWAS SNPs (105). Thus, cytokine-mediated transcriptional regulation of ASM contractile responses has a profound impact on the asthma phenotype.

Therapeutic opportunities to reduce ASM solidification and/or enhance ASM fluidization in asthma

Studies of cytoskeletal mechanisms in ASM have uncovered new therapeutic targets for the treatment of AHR by either enhancing fluidization or reducing resolidification (Table 2). At the receptor level, for example, α-adrenergic receptor expression has been documented in HASM (106). Epinephrine, an α- and β-adrenergic agonist used for severe asthma exacerbations, induces contraction of β-agonist desensitized HASM cells in a α1-adrenergic receptor–dependent fashion. This phenotypic switch may partially explain why prolonged β-agonist and β-antagonist therapy often worsens bronchospasm and suggests a role for α-adrenergic receptor antagonists to partially fluidize ASM cells that are in a refractory solidified state during acute asthma exacerbations.

An inhibitor of Gαq (FR900359) nearly abolishes HASM solidification by inhibiting Ca^{2+} flux and airway contraction in PCLS. Inhalation of FR900359 strongly prevents MCh-induced bronchoconstriction in allergen-challenged mice with little to no effects on airway inflammation or systemic side effects (107). Further downstream of Gαq, DAG is degraded to phosphatidic acid by DAG kinase ζ (DGKζ) in ASM. Administration of a DGK inhibitor (R59949) to mice reduces AHR and CCh-evoked PCLS airway constriction (108). DGK inhibition in HASM results in an increased DAG–phosphatidic acid ratio, which in turn prevents Gαq-mediated PLCβ activation in a feedback loop (109). Notably, administration of R59949 to mice also impairs cytokine production by T helper type 2 cells (108), thereby reducing their effects on ASM solidification pathways.

Various PI3K isoforms are also required for airway contraction. A selective inhibitor of PI3Kγ weakens ACh-induced mouse PCLS contraction by inhibiting Ca^{2+} oscillations (110). Another study demonstrated that siRNA-mediated PI3Kδ knockdown or pharmacological inhibitors reduces CCh-induced contraction of human PCLS and elicits relaxation even in β-agonist desensitized airways (111). These interventions impaired CCh-induced solidification mediated by MLC phosphorylation.

RhoA–ROCK expression is increased in Th2 cytokine (IL4/13)-treated HASM and allergen-challenged mice. Inhalation of ROCK inhibitors such as Y27632 and fasudil inhibits the development of allergen-induced AHR by reducing ASM contraction and airway inflammation (112). ROCK inhibition can also synergistically enhance stretch-induced ASM fluidization (113). Newer generation ROCK inhibitors such as netarsudil are used safely in patients with glaucoma to treat intraocular hypertension (114), suggesting that inhaled versions of these drugs might be developed for asthma treatment.

Statins including pitavastatin and atorvastatin, which are widely used to reduce serum cholesterol levels, have emerged

as potential therapy for asthma (115). By inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme of sterol biosynthesis in the mevalonate pathway, statins deplete membrane pools of the metabolite geranylgeranyl pyrophosphate. This process reduces geranylgeranylation-mediated localization of RhoA at the PM (116). Treatment of PCLS with pitavastatin weakens contraction in unstimulated and agonist (histamine and MCh)-stimulated airways *ex vivo*. Pitavastatin counteracts solidification by means of reduced MLC phosphorylation and F-actin formation. Under a dynamic stretch environment, pitavastatin also promotes ASM fluidization (116). Inhalation of atorvastatin impairs development of allergen-induced AHR in mice through multiple mechanisms including reduced inflammation and remodeling (117). A retrospective study of patients visiting Ajou University Medical Center in Korea found that taking statins for dislipidemia deceased the risk for severe and nonsevere asthma exacerbations (118).

Other compounds that potentiate stretch-induced ASM fluidization or inhibit stretch-induced ASM resolidification by affecting actin polymerization might be developed, including those targeting zyxin, cofilin, or Abl, or actin–myosin connectivity (119). These drugs may complement conventional therapies such as β-agonists, which do not affect this process (116). For example, activation of the bitter taste receptor AS2R14 elicits airway relaxation by promoting cofilin-mediated destabilization of F-actin (120). Overall, ASM cytoskeleton–targeted drugs demonstrate promise for the treatment of AHR.

Conclusions

The concept of ASM hypercontraction as an essential cause of airway obstruction in all asthma endotypes is supported by the rapid and sustained clinical improvement associated with ASM ablation strategies (bronchial thermoplasty) (121). Recent investigations have uncovered previously unrecognized extrinsic factors that augment ASM contraction, including ECM stiffness and cell–cell coupling (122), obesity (123, 124), and epithelial crosstalk (125). Conversely, these studies also revealed that persistent bronchospasm may itself propagate inflammation by eliciting epithelial extrusion from the airway and lung damage (126).

To understand these emerging concepts, it is imperative to view ASM contraction not as a static state but as a continuously adapting dynamic process that proceeds over a time scale of seconds to minutes and is inescapable, ever-present, and dominant in the living breathing lung (127). This dynamic process conforms to the phenomenological description of fluidization and resolidification in inorganic materials, with supportive evidence gleaned from multiple levels—contractile proteins, the ASM cell in culture, the muscle strip, the ASM within the bronchial airway, and the lung. We emphasize that this physical description is interdependent with molecular effectors and modulators of ASM contraction, including both GPCR-mediated and actin-mediated contractile and relaxation pathways. Neither does it minimize the effects of mediators, cytokines, and genetic contributions. It does, however, set strict physical expectations to biochemical changes. Unravelling this complexity will advance our understanding of contractile mechanisms.

The evidence that ASM cells cultured from patients with asthma solidify more effectively in response to contractile mediators is conflicting (128). The contribution of autonomous defects in cytoskeletal protein expression and/or function to ASM hypercontraction in chronic established asthma also requires further investigation. Recent studies of the biomechanical properties of ASM cells cultured from subjects with asthma (23) and a proteomic study of intrapulmonary airways (129) revealed hypercontractility in diseased samples. The phenotype was associated with upregulation of zyxin and the ASM-specific protein smoothelin, among others. Further proteogenomic and functional examination of the smaller airways from patients with asthma in their native environment (*e.g.*, PCLS or organoids) will advance our understanding of contractile mechanisms and no doubt uncover previously unrecognized therapeutic targets.

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Abbreviations—The abbreviations used are: ACh, acetylcholine; AHR, airway hyper-responsiveness; ASM, airway smooth muscle; CCh, carbachol; DAG, diacylglycerol; DGK, DAG kinase; ECM, extracellular matrix; FAK, focal adhesion kinase; GMFγ, glia maturation factor γ; GPCR, G protein-coupled receptor; GWAS, genome-wide association study; HASM, human ASM; ILK, integrin-linked kinase; IL-31R, IL-31 receptor; MCh, methacholine; MLCK, myosin light chain kinase; NM, nonmuscle; PAO, phenylarsine oxide; PCLS, precision cut lung slice; PM, plasma membrane; ROCK, Rho-associated coiled-coil forming kinase; SR, sarcoplasmic reticulum; TGFβ, transforming growth factor beta.

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