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Interaction between endoplasmic/sarcoplasmic reticulum stress (ER/SR stress), mitochondrial signaling and Ca^{2+} regulation in airway smooth muscle (ASM)1

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Abstract

Airway inflammation is a key aspect of diseases such as asthma. Several inflammatory cytokines (e.g., TNFα and IL-13) increase cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) responses to agonist stimulation and Ca^{2+} sensitivity of force generation, thereby enhancing airway smooth muscle (ASM) contractility (hyper-reactive state). Inflammation also induces ASM proliferation and remodeling (synthetic state). In normal ASM, the transient elevation of $[Ca^{2+}]_{cyt}$ induced by agonists leads to a transient increase in mitochondrial Ca^{2+} ($[Ca^{2+}]_{mito}$) that may be important in matching ATP production with ATP consumption. In human ASM (hASM) exposed to TNFα and IL-13, the transient increase in $[Ca^{2+}]_{mito}$ is blunted despite enhanced $[Ca^{2+}]_{cyt}$ responses. We also found that TNFα and IL-13 induce reactive oxidant species (ROS) formation and endoplasmic/sarcoplasmic reticulum (ER/SR) stress (unfolded protein response) in hASM. ER/SR stress in hASM is associated with disruption of mitochondrial coupling with the ER/SR membrane, which relates to reduced mitofusin 2 (Mfn2) expression. Thus, in hASM it appears that TNFα and IL-13 result in ROS formation leading to ER/SR stress, reduced Mfn2 expression, disruption of mitochondrion–ER/SR coupling, decreased mitochondrial Ca^{2+} buffering, mitochondrial fragmentation, and increased cell proliferation.

Keywords

inflammation; ER/SR stress; airway; asthma; unfolded protein response

Introduction

Asthma affects more than 20 million people in the USA (~10% of the population), and more than 300 million people world-wide (GINA 2012). It is now recognized that inflammation is key to asthma pathophysiology, and involves mediators such as the cytokines tumor necrosis factor alpha (TNFα) and interleukin 13 (IL-13). Yet, therapies that blunt inflammation per se are not entirely effective. While a number of cell types such as airway epithelium and immune cells are likely to play some role in asthma, airway smooth muscle (ASM) is the

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key player by contributing to both exaggerated airway narrowing via enhanced cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and Ca^{2+} sensitivity of force responses to agonist (e.g., acetylcholine, ACh) stimulation (i.e., "hyper-reactive" state) and airway remodeling via increased ASM cell proliferation and the formation of extracellular matrix (ECM) (i.e., "synthetic" state) (Fig. 1) (James 2005; Joubert and Hamid 2005; Black et al. 2012; Prakash 2013; Wright et al. 2013*a*, 2013*b*). Thus, it appears that in asthmatics, ASM exists in both hyper-reactive and synthetic states, thus contributing to a thicker, more responsive airway. Accordingly, upstream mechanisms that promote both hyper-reactive and synthetic states of ASM, particularly under the influence of inflammation, represent an appealing target for therapeutic intervention.

Relationship between elevated $[Ca^{2+}]_{cyt}$ and force in ASM

Force generation in ASM is initiated by a transient increase in $[Ca^{2+}]_{cyt}$ in response to agonist (e.g., ACh) stimulation (Sieck et al. 2001). The mechanisms underlying the transient increase in $[Ca^{2+}]_{cyt}$ are complex, but involve Ca^{2+} influx and (or) Ca^{2+} release from the endoplasmic/sarcoplasmic reticulum (ER/SR). Based on available evidence, it is unlikely that depolarization-dependent Ca^{2+} influx is required for the immediate $[Ca^{2+}]_{cyt}$ response to agonist stimulation in ASM (Kannan et al. 1997; Prakash et al. 1997). However, Ca^{2+} influx in ASM does have an important role in establishing basal $[Ca^{2+}]_{cyt}$ levels (Wylam et al. 2012) as well as in replenishing depleted SR Ca^{2+} stores via store-operated Ca^{2+} entry (SOCE) (Ay et al. 2004, 2006; White et al. 2006; Sieck et al. 2008; Sathish et al. 2012; Jia et al. 2013).

The transient elevation of $[Ca^{2+}]_{cyt}$ in ASM cells is mediated in part by the production of inositol 1,4,5-triphosphate (IP_3), which activates IP_3 receptors to allow SR Ca^{2+} release (Foskett et al. 2007; Jude et al. 2008; Bai et al. 2009; Mei et al. 2014). The initial release of SR Ca^{2+} is amplified by the Ca^{2+}-induced activation of additional IP_3 receptors as well as ryanodine receptors (RyR); a process called Ca^{2+}-induced Ca^{2+} release (CICR) (Mei et al. 2014). In ASM, the open probability of the RyR channels and CICR is also enhanced by agonist-induced production of cyclic ADP ribose (Kannan et al. 1996; Prakash et al. 1998; White et al. 2006).

The SR Ca^{2+} stores are replenished via the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Sathish et al. 2008, 2009; Prakash et al. 2009). In ASM cells, the interaction between SR Ca^{2+} release via IP_3 receptor and ryanodine receptor (RyR) channels and SR Ca^{2+} uptake via SR calcium transport ATPase (SERCA) is dynamic, resulting in localized $[Ca^{2+}]_{cyt}$ oscillations (~800–1000 nmol·L^{-1} amplitude at frequencies ranging from ~5–40 min^{-1}) that propagate through the cell (15–25 μm^{-1}) (Prakash et al. 1997, 2000; Sieck et al. 1997, 2001). Both the frequency and propagation velocity of $[Ca^{2+}]_{cyt}$ oscillations in ASM cells depend on ACh concentration, whereas the amplitude of the localized $[Ca^{2+}]_{cyt}$ response is independent of ACh concentration (Prakash et al. 1997, 2000; Sieck et al. 1997, 2001) Thus, the ACh concentration dependence of the global $[Ca^{2+}]_{cyt}$ response in ASM cells is based on the frequency modulation of local responses and their fusion into larger responses.

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In ASM cells, there is a delay of several hundred milliseconds between the agonist-induced elevation of $[Ca^{2+}]_{cyt}$ and the resulting mechanical response (Sieck et al. 2001). This time delay reflects a cascade of events underlying excitation–contraction coupling in ASM that can be summarized into 4 main steps: (*i*) mobilization of calmodulin (CaM); (*ii*) activation of myosin light chain kinase (MLCK) following Ca^{2+} binding to CaM; (*iii*) MLCK-mediated phosphorylation of MLC_{20}; and (*iv*) cross-bridge recruitment and cycling (Sieck et al. 2001). It has been shown that mobilization of CaM in ASM introduces the longest delay in excitation–contraction coupling in ASM, while delays owing to MLC20 phosphorylation, cross-bridge recruitment, and cycling are minimal (Sieck et al. 2001). The level of MLC_{20} phosphorylation also depends on myosin light chain phosphatase (MLCP), and the Ca^{2+} sensitivity of ASM force generation is modulated by the balance between MLCK and MLCP activities (Sieck et al. 2001). In ASM, agonist stimulation increases Rho-associated protein kinase (ROCK), which inhibits MLCP activity, thereby mediating a Ca^{2+} independent pathway for modulating ASM force generation (Ca^{2+} sensitivity). Agonist-induced increase in ROCK activity also indirectly mediates cyto-skeletal remodeling in ASM via inhibition of actin filament depolymerization.

Actin/myosin interactions in ASM

The essential molecular mechanism underlying force generation and contraction in ASM, like all muscle, is based on the Ca^{2+}-regulated binding of myosin to actin to form cross-bridges. Force generation in ASM depends on the number of cross-bridges recruited in parallel, whereas the velocity of shortening (contraction) depends on the cycling of cross-bridges from bound to unbound states. In skeletal and cardiac (striated) muscle, myosin filaments form α-helical, bipolar configuration, whereas in smooth muscle, monomeric myosin is assembled into polymers or thick filaments in a nonhelical, side-polar configuration (Hodgkinson et al. 1995; Xu et al. 1996). The bipolar arrangement of myosin in skeletal and cardiac muscle together with the steric constraints of the sarcomeric structure impose limits on muscle length and shortening that do not exist in smooth muscle (Xu et al. 1996; Seow 2005). In striated muscle, actin filaments are anchored to the Z-disks of the sarcomere for longitudinal transmission of force. In contrast, actin filaments in smooth muscle are clustered into regions called dense bodies or membrane-associated dense plaques that allow force transmission to the ECM only when anchored to the plasma membrane (Hodgkinson et al. 1995; Gunst and Tang 2000; Kuo et al. 2003*a*; Sieck and Gransee 2012). Importantly, the internal arrangement of actin/myosin filaments, dense bodies, and dense plaques in smooth muscle is not highly ordered as in striated muscle, and this arrangement undergoes remodeling during the contractile response (Mehta and Gunst 1999; Gunst et al. 2003; Herrera et al. 2004; Seow 2005; Gunst and Zhang 2008; Zhang and Gunst 2008).

Actin exists in 2 distinct but interchangeable forms: polymeric, filamentous actin (F-actin) and monomeric actin (G-actin) (Jones et al. 1999*b*; Mehta and Gunst 1999; Gunst and Zhang 2008). During ASM activation, there is a decrease in G-actin content indicating that actin cytoskeletal remodeling and inhibition of actin polymerization reduces isometric force generation (Jones et al. 1999*b*; Mehta and Gunst 1999; Gunst et al. 2003). In addition to actin polymerization, there is also evidence that the contractile network is remodeled through myosin polymerization (Kuo et al. 2001, 2003*a*, 2003*b*; Seow 2005; Ali et al. 2007;

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Ijpma et al. 2011), such that the length of the myosin filaments increases, thereby increasing the number of myosin heads that can potentially form cross-bridges. In ASM, focal adhesion complexes (membrane-bound dense plaques) couple F-actin to the ECM (Gunst et al. 2003). The focal adhesion complexes comprise a scaffold of structural and signaling proteins that include mechano-sensitive transmembrane integrins (Gunst and Zhang 2008; Zhang and Gunst 2008). It is possible that in ASM, the location of focal adhesion complexes on the plasma membrane are dynamically regulated such that they recruit actin polymerization (increasing internal loading) according to local mechanical stimuli experienced during contraction (Gunst and Zhang 2008; Zhang and Gunst 2008). External loading of ASM is complex and dynamic, varying with inflation and deflation of the lung (Gunst et al. 2003; Gunst and Zhang 2008; Zhang and Gunst 2008). Thus, the internal organization of the actin/myosin filament lattice in ASM is likely to be complex and dynamic as well, changing in response to internal cell signaling and external loading (Gunst and Tang 2000; Gunst et al. 2003; Kuo et al. 2003*a*; Zhang and Gunst 2008).

Cytokine enhancement of $[Ca^{2+}]_{cyt}$ and force responses in ASM: hyper-reactivity

As mentioned above, one hallmark of asthma is ASM hyper-reactivity in response to agonist (e.g., ACh) stimulation. This effect on ASM force generation and contractility is mimicked in animal models following airway sensitization (Stephens et al. 2003; Aravamudan et al. 2012), or following cytokine exposure in ASM muscle strips (Fig. 2), or in lung slice preparations (Fig. 3) (Amrani 2014). The increase in ASM force generation is the consequence of both an increased $[Ca^{2+}]_{cyt}$ response to agonist stimulation as well as an increase in the Ca^{2+} sensitivity of force generation. We and others have demonstrated that inflammatory cytokines enhance agonist-induced $[Ca^{2+}]_{cyt}$ responses in ASM (Parris et al. 1999; Amrani et al. 2000; Reynolds et al. 2000; Amrani and Panettieri 2002; Chen et al. 2003; Hunter et al. 2003; Sakai et al. 2004; White et al. 2006; Tirumurugaan et al. 2007; Sathish et al. 2008, 2009, 2011*a*, 2011*b*; Delmotte et al. 2012). Several major mechanisms involved in Ca^{2+} homeostasis and agonist-induced $[Ca^{2+}]_{cyt}$ responses are modulated by cytokines (Jude et al. 2008; Sanderson et al. 2008; Prakash 2013; Wright et al. 2013*c*; Amrani 2014; Delmotte et al. 2014; Matsumoto 2014).

Mechanical work and ATP hydrolysis in ASM

In all muscle types, cross-bridge recruitment and cycling underlie the mechanical work performed through force generation and contraction. To perform this mechanical work, muscle requires chemical energy in the form of ATP hydrolysis (actin-activated myosin ATPase). The energy released by ATP hydrolysis is coupled to the intrinsic force development and load displacement that is mediated by cyclic attachment and detachment of myosin heads to actin (cross-bridge cycling). In skeletal muscle fibers, isometric force generation at any given level of Ca^{2+} activation is accompanied by a sustained rate of ATP hydrolysis; thus, tension cost (ATP consumed per unit of force) does not change (Sieck et al. 1998). In contrast, during sustained isometric force generation in ASM, the rate of ATP hydrolysis decreases and as a result tension cost decreases with time (Fig. 4) (Sieck et al. 1998; Jones et al. 1999*a*, 1999*b*). The time-dependent decrease in rate of ATP hydrolysis in

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ASM is also associated with a slowing of shortening velocity and has been termed the “latch state” (Hai and Murphy 1988; Murphy and Rembold 2005). According to the “latch bridge” hypothesis, a unique cross-bridge state occurs when the myosin head of an attached cross-bridge (due to prior MLC_{20} phosphorylation) is then dephosphorylated. These dephosphorylated latch-bridges cycle at a slower rate with an increased duty cycle (amount of time attached versus detached), and therefore maintaining force at a reduced energy cost. The main assumption of the latch-bridge model is that the extent of MLC_{20} phosphorylation determines the “latch” or energetic state of smooth muscle force generation (Hai and Murphy 1988; Murphy and Rembold 2005). However, in permeabilized canine ASM, we (Jones et al. 1999*a*) showed that with sustained maximum Ca^{2+} activation, isometric force and the level of MLC_{20} phosphorylation are sustained, while the rate of ATP hydrolysis decreases with time reflecting a reduction in energy cost, consistent with a latch state, but without a change in MLC_{20} phosphorylation (Fig. 4). Furthermore, when dephosphorylation of MLC_{20} was prevented by thiophosphorylation (using ATPγS), the time-dependent decrease in ATP hydrolysis rate following Ca^{2+} activation was unaffected. Thus, the slowing of cross-bridge cycling rate and ATP hydrolysis rate in ASM is independent of any change in the level of MLC_{20} phosphorylation. Alternatively, we proposed that the decline in the rate of ATP hydrolysis in ASM during sustained Ca^{2+} activation may be due to an increase in “internal loading” as a result of cytoskeletal remodeling (Jones et al. 1999*b*). This alternative “internal loading” hypothesis was tested by inhibition of actin remodeling with phalloidin (which binds F-actin, preventing its depolymerization), which essentially eliminated the time-dependent decrease ATP hydrolysis rate in ASM (Fig. 4) (Jones et al. 1999*b*). According to this internal loading hypothesis, cross-bridges in ASM cells are recruited by MLC_{20} phosphorylation, but a proportion of the cross-bridges are not fully attached to the internal cytoskeletal structure, resulting in lower internal loading, faster cross-bridge cycling, and a high rate of ATP hydrolysis. Subsequently, with cytoskeletal remodeling, there is an increase in cross-bridge attachment to the cytoskeleton imposing increased internal loading, a slowing of cross-bridge cycling rate, and a decrease in ATP hydrolysis rate.

Metabolic cost of cytokine-induced hyper-reactivity in ASM

In previous studies in ASM (Sieck et al. 1998; Jones et al. 1999*a*, 1999*b*), we demonstrated a direct relationship between ATP hydrolysis rate and force generation (Fig. 4), velocity of shortening, and power output. Thus, in ASM exposed to inflammatory cytokines, the increase in force generation is associated with an increase in ATP consumption. In porcine ASM, maximum ATP hydrolysis occurs at peak power output, attaining a rate of ~0.12 nmol $ATP \cdot mm^{-3} \cdot s^{-1}$ that is approximately 3-fold higher than the ATP hydrolysis rate during maximum isometric force (~0.04 nmol $ATP \cdot mm^{-3} \cdot s^{-1}$) (Sieck et al. 1998). By comparison, in fast-twitch diaphragm muscle fibers, the rate of ATP hydrolysis is ~1.2 nmol $ATP \cdot mm^{-3} \cdot s^{-1}$ during maximum isometric force and increases to ~2.7 nmol $ATP \cdot mm^{-3} \cdot s^{-1}$ during peak power output (Sieck et al. 1998). Tension cost ([ATP] consumed per force generated) in diaphragm muscle fibers ranges from ~16 000–35 000 nmol $ATP \cdot s^{-1} \cdot mN^{-1}$ depending on fiber type. In contrast, the tension cost of ASM is 6- to 14-fold lower at ~2500 nmol $ATP \cdot s^{-1} \cdot mN^{-1}$ (Sieck et al. 1998). Of course these differences reflect the mechanical/

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work performance of these different muscle types, e.g., the maximum power output of skeletal muscle is ~10 to 100 times greater than that of ASM (Sieck et al. 1998). In skeletal muscle fibers this greater energy demand is met by a relatively large pool of phosphocreatine (PCr) that is available to buffer the transient increase in ATP hydrolysis (Paul 1989). Smooth muscle contains a very small PCr pool, with a much greater relative dependence on ATP production rather than PCr buffering (Krisanda and Paul 1983; Spurway and Wray 1987; Ishida et al. 1994). Thus, skeletal muscle mechanical performance is optimized for maximum power output, while the mechanics of smooth muscle is optimized for maximum energy efficiency. Efficient matching of energy supply with energy demand may be an even more significant governing factor for the ASM contractile response.

In all muscle types, mitochondria provide ATP through O_2 consumption and oxidative phosphorylation to meet the energy demands of force generation and contraction. In vascular smooth muscle, it has been shown that isometric force is strongly correlated with O_2 consumption (Krisanda and Paul 1983; Paul 1989; Ishida et al. 1994). It is likely that the same relationship holds for ASM. Thus, with an inflammatory-cytokine-induced increase in force and contractile response and the associated increase in ATP consumption, it is likely that O_2 consumption and oxidative phosphorylation also increase (Fig. 5).

Mitochondrial ATP production is stimulated by an increase in mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$) via an increase in activity of Ca^{2+}-sensitive intra-mitochondrial dehydrogenases associated with the tricarboxylic acid (TCA) cycle – pyruvate dehydrogenase (PDH), NAD-isocitrate dehydrogenase (ICDH), and oxoglutarate dehydrogenase (OGDH) (Denton and McCormack 1990; Denton 2009; Griffiths and Rutter 2009; Glancy and Balaban 2012; Tarasov et al. 2012). The relationship between dehydrogenase activity and $[Ca^{2+}]_{mito}$ can also be reversed by an excess of end-product (for example, excess acetyl-CoA in the case of PDH), and can also be modulated by the ratio of ATP/ADP (which decreases during muscle contraction and increased ATP consumption) and substrate availability within the mitochondria (Denton 2009).

In the process of oxidative phosphorylation, NADH donates electrons to the electron transport chain (ETC) within the inner membrane of the mitochondria. As the ETC catalyzes electron flow, H^+ is pumped against its concentration gradient out of the mitochondrial matrix. The final stage of the ETC requires O_2 to accept electrons (at Complex IV). Therefore, electrons flowing through the ETC results in O_2 consumption and, owing to H^+ pumping, the formation of a proton motive force (pmf) that has electrical ($\Delta\Psi$) and chemical (ΔpH) features. The pmf favors the flow of protons into the mitochondrial matrix. The F_1F_o-ATPase (also called Complex V), which is permeable to H^+, uses the energy dissipated by pmf-driven proton flow into the matrix to synthesize ATP from ADP and inorganic phosphate (Pi). Synthesized ATP is then transported to the cytosol in exchange for cytosolic ADP by the adenine nucleotide transporter (ANT). Normally, the O_2 consumed in the ETC is reduced to produce water. However, approximately 1% of O_2 is incompletely reduced and yields the superoxide radical (O_2^-). Thus, reactive oxidant species (ROS) formation is a natural by product of oxidative phosphorylation. However, metabolic stress can increase ROS formation, triggering oxidative stress in cells (Fig. 5).

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Cytokine-induced ER/SR stress and the unfolded protein response in ASM

A major feature of asthma is an increase in ROS formation (Katsumata et al. 1990; Comhair and Erzurum 2010; Zuo et al. 2013). In other cell systems, it has been shown that one consequence of excessive ROS formation is an accumulation of unfolded or misfolded proteins in the ER/SR lumen, which triggers a highly conserved restorative/corrective response (i.e., the ER/SR stress or unfolded protein response) (Adolph et al. 2012; Baban et al. 2012; Garg et al. 2012; Hasnain et al. 2012*a*, 2012*b*; Martino et al. 2012). As a normal physiological process, the ER/SR stress response serves to restore normal function by halting protein translation and activating protein degradation and expression of molecular chaperones involved in protein folding (Welihinda et al. 1999; Fulda et al. 2010; Adolph et al. 2012; Wang and Kaufman 2012). Three ER/SR stress protein markers located at the ER/SR membrane are involved in the unfolded protein response: protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1α) (Fig. 6). Normally, the natural molecular chaperone protein binding immunoglobulin protein (BiP) (also known as 78 kDa glucose-regulated protein (GRP)-78 or 70 kDa heat shock protein 5 (HSPA5)) is associated with PERK, ATF6, and IRE1 and maintains these proteins in an inactive state. However, as unfolded proteins accumulate in the ER/SR lumen, expression of BiP/GRP-78 increases and it dissociates from PERK, ATF6, and IRE1 leading to the activation of these ER/SR stress proteins and the downstream pathways they mediate. IRE1α catalyzes the alternative splicing of X-box binding protein 1 (XBP1) mRNA leading to expression of the active (spliced) XBP1 transcription factor. ATF6 translocates to the golgi and is cleaved by the proteases S1P and S2P, leading to an active ATF6 transcription factor ATF6 (i.e., ATF6p50). PERK phosphorylates the translation-initiator factor eukaryotic initiation factor 2 (eIF2α), leading to selective translation of the transcription factor ATF4. The IRE1, ATF6, and PERK pathways, and thereby XBP1, ATF6p50, and ATF4 act to: (*i*) block protein translation; (*ii*) increase chaperone expression (e.g., calnexin); and (*iii*) enhance SR-associated protein degradation pathways (Fig. 6) (Samali et al. 2010; Munoz and Zorzano 2011; Adolph et al. 2012; Baban et al. 2012; Garg et al. 2012; Dromparis et al. 2013).

A recent study (Makhija et al. 2013) showed that there is an increased expression of ER/SR stress protein markers such as BiP/GRP-78 and ATF6 in lungs from an ovalbumin (OVA) sensitized mouse model of asthma, and that chemical chaperone treatment used to attenuate ER/SR stress is effective in reducing airway remodeling and inflammation. Others recent studies confirmed the presence of ER/SR stress markers in the OVA mouse model (Kim et al. 2013) as well as another mouse model of asthma: house dust mite sensitization (Hoffman et al. 2013). However, the involvement of ASM per se, and the downstream mechanisms by which ER/SR stress influences airway reactivity and remodeling were not explored in these studies.

In a recent unpublished study, we confirmed that exposure to inflammatory cytokines (TNFα or IL13) induces ROS formation and ER/SR stress in hASM cells. In these studies, hASM cells were acutely dissociated from >3rd order bronchi (~3 mm diameter) from normal human lung biopsy tissue obtained during thoracic surgery. The hASM cells were exposed to TNFα or IL-13 (20 ng/mL) for 24 h (n = 3 ASM samples per group). The

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response to tunicamycin (1.0 μg/mL) served as a positive control for ER/SR stress. The induction of ER/SR stress was verified by increased expression (determined by Western blot) of BiP/GRP78 (upstream marker), IRE1, ATF6, and PERK (ER/SR stress markers), as well as spliced XBP1 and calnexin (downstream markers). ER/SR stress was also confirmed by the increased ratio of spliced versus unspliced XBP1 mRNA measured using real-time reverse-transcription PCR, which peaked at 12 h of cytokine exposure.

Cytokine-induced disruption of mitochondrial coupling with ER/SR membrane in ASM

Within ASM cells, the ER/SR and mitochondria are discreet intracellular organelles. However, both the ER/SR and mitochondria are highly dynamic, moving and undergoing constant structural and spatial reorganization (see below). Physical contact between the ER/SR membrane and the outer mitochondrial membrane (e.g., mitochondria-associated ER/SR membrane, MAM) is especially important for efficient transfer of metabolites (lipids, Ca^{2+}) between the 2 organelles and is essential for their normal function (de Brito and Scorrano 2010; Kornmann 2013; Rainbolt et al. 2014). Disruption of the normal coupling between mitochondria and the ER/SR membrane appears to be an important downstream consequence of ER/SR stress (Bravo et al. 2012; Verfaillie et al. 2012; Vannuvel et al. 2013; Rainbolt et al. 2014). In addition, PERK and another ER/SR protein, ER oxidoreductin 1α (Ero1α), are associated with MAMs, and they appear to leave MAMs when cells experience ER/SR stress (Gilady et al. 2010; Raturi and Simmen 2013). While the role of PERK and Ero1α within MAMs is unclear, it suggests that uncoupling of mitochondria with the ER/SR membrane may be an important component of the overall ER/SR stress response. In this regard, mitofusin-2 (Mfn2), which is well known for its role in the regulation of mitochondria morphology (see below), is also an important component of MAMs, involved in the formation of bridges between mitochondria and the ER/SR membrane (de Brito and Scorrano 2008*a*, 2008*b*, 2010). Mfn2 protein expressed on the ER/SR membrane forms homotypic and heterotypic complexes with Mfn2 and Mfn1 proteins present on the outer mitochondrial layer (Fig. 7). Interestingly, the ER/SR stress marker PERK co-localizes with Mfn2 protein within MAMs (Verfaillie et al. 2012; Munoz et al. 2013). Ablation of the Mfn2 gene enhances ER/SR stress, while ablation of the PERK gene increases Mfn2 expression suggesting a homeostatic interaction between Mfn2 and PERK expression (Munoz et al. 2013). However, the exact mechanisms are unknown. In a recent study, we reported that Mfn2 expression is decreased in asthmatic ASM (Aravamudan et al. 2014); however, the relationship between reduced Mfn2 expression and mitochondrial association with the ER/SR membrane was not explored. More recently, we found that in hASM cells exposed to TNFα or IL13, Mfn2 expression was markedly reduced, whereas PERK expression increased, consistent with the ER/SR stress response. We also found that following 24 h exposure of hASM to TNFα or IL-13, the normal enhancement of mitochondria proximity to the ER/SR induced by ACh stimulation was blunted. These results suggested that the normal coupling between mitochondria and the ER/SR membrane was disrupted in hASM cells as a result of ER/SR stress.

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Cytokine-induced reduction of mitochondrial movement/motility in ASM

Mitochondrial movement or motility is another potent regulator of the coupling between mitochondria and the ER/SR membrane. Movement of mitochondria toward the ER/SR should promote MAM formation. A redistribution of mitochondria to areas of high $[Ca^{2+}]_{cyt}$ has been reported (Yi et al. 2004; Anesti and Scorrano 2006; Boldogh and Pon 2007), and agonist stimulation increases mitochondrial density near the ER/SR (Brough et al. 2005; Malaiyandi et al. 2005). Mitochondria movement is also critical to maintain normal mitochondrial morphology (see below), and therefore, mitochondrial function and integrity. It is not clear whether ER/SR stress affects mitochondrial movement. In hASM, there is no information regarding the effect of inflammation on mitochondrial movement. Similarly, there is no information whether mitochondrial movement is impaired in airway diseases such as asthma.

In a recent study (Zavaletta et al. 2011), we observed 2 general types of mitochondrial movement in hASM cells: (*i*) a continuous apparent random, non-directed movement that occurs over relatively short distances, and (*ii*) an infrequent directed trajectory movement that is most apparent in the distal compartment of the cell. In hASM cells we found that agonist (ACh, histamine) stimulation reduced the random, non-directed movement of mitochondria, whereas the directed trajectory movement of mitochondria in distal compartments of the cell appeared to increase. We also found that 24 h exposure of hASM cells to TNFα decreases both types of mitochondrial movement. It is possible that the movement of mitochondria toward the ER/SR in hASM cells is essential for MAM formation and coupling of mitochondria to the ER/SR membrane (Fig. 7).

Cytokine-induced disruption of coupling between $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mito}$ in ASM

In many cell types, including hASM, a transient elevation of $[Ca^{2+}]_{cyt}$ leads to a coupled transient increase in mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$) mediated via Ca^{2+}($[Ca^{2+}]_{cyt}$)-induced activation of the mitochondrial Ca^{2+} uniporter (MCU) (Fig. 8) (Baughman et al. 2011; De Stefani et al. 2011; Delmotte et al. 2012, 2014; Raffaello et al. 2012). However, the MCU is relatively insensitive to Ca^{2+}, requiring $[Ca^{2+}]_{cyt}$ levels that are much higher (>10 μM Ca^{2+}) than normally encountered in the bulk cytosol during agonist stimulation ($[Ca^{2+}]_{cyt}$ is typically <2.0 μmol·L^{-1} Ca^{2+}) (Gunter and Sheu 2009). This high threshold for MCU activation (>10 μmol·L^{-1} Ca^{2+}) compared with the lower subthreshold bulk $[Ca^{2+}]_{cyt}$ is reconciled by the “hotspot hypothesis”, which presumes that the cytosol in the immediate vicinity of an open Ca^{2+} channel (either inositol triphosphate (IP_3) or RyR) can transiently experience intense increases in $[Ca^{2+}]_{cyt}$ (Fig. 7) (Rizzuto et al. 2004). Interestingly, both IP_3Rs and RyRs have been identified within MAMs (Hajnoczky et al. 2002; Kiviluoto et al. 2013). A Ca^{2+} hotspot thereby exposes the MCU to a microdomain of high $[Ca^{2+}]_{cyt}$ sufficient for MCU activation and a coupled increase in $[Ca^{2+}]_{mito}$ (Fig. 8) (Rizzuto et al. 1993, 2004, 2009; Rizzuto and Pozzan 2006; Pizzo et al. 2012) Thus, mitochondrial coupling with the ER/SR membrane is essential for MCU activation and the transient elevations of $[Ca^{2+}]_{mito}$ that normally are coupled to transient $[Ca^{2+}]_{cyt}$ responses (Fig. 8). Recent studies reported that ER/SR stress is associated with reduced mitochondrial

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coupling with the ER/SR membrane, resulting in changes in $[Ca^{2+}]_{mito}$ (Csordas et al. 2006; Raturi and Simmen 2013). In various models of ER/SR stress, expression of Mfn2 is altered and this is correlated with uncoupling of mitochondria with the ER/SR membrane and blunting of the transient $[Ca^{2+}]_{mito}$ response. Ablation of the Mfn2 gene in mouse embryonic fibroblasts and HeLa cells reduces $[Ca^{2+}]_{mito}$ in a manner similar to direct MCU inhibition of mitochondrial Ca^{2+} uptake (de Brito and Scorrano 2008*a*). We also found that 24 h exposure of hASM cells to TNFα and (or) IL-13 resulted in reduced Mfn2 expression, reduced proximity of mitochondria to the ER/SR, and a marked reduction in the transient $[Ca^{2+}]_{mito}$ response to ACh stimulation, even though the transient $[Ca^{2+}]_{cyt}$ response was enhanced (Delmotte et al. 2012).

The concept that mitochondria could compete with the SR to buffer $[Ca^{2+}]_{cyt}$ is controversial (Collins et al. 2000; Cheranov and Jaggar 2004; Ishii et al. 2006; Chalmers and McCarron 2008, 2009). However, mitochondria have a huge Ca^{2+} buffering capacity and, importantly, are often strategically located near IP_3 or RyR Ca^{2+} channels (Hajnoczky et al. 2002; Kiviluoto et al. 2013). It is presumed that mitochondrial Ca^{2+} uptake and (or) release in proximity to IP_3 or RyR Ca^{2+} channels may affect local $[Ca^{2+}]_{cyt}$ and thereby their probability of opening. In addition, changes in $[Ca^{2+}]_{mito}$ affect mitochondrial function (i.e., ATP synthesis, ROS generation), which in turn could affect nearby Ca^{2+} channels (Perocchi et al. 2010; Delmotte et al. 2014). It has been suggested that ROS increase the “open probability” of RyR channels (Favero et al. 1995). Several studies showed that endogenous ROS lead to an elevation in $[Ca^{2+}]_{cyt}$ (Lin et al. 2007; Chen et al. 2010; Perez-Vizcaino et al. 2010), which is blunted by the inhibition of RyR channels (Lin et al. 2007). When mitochondrial Ca^{2+} uptake (via MCU) is inhibited in ASM cells, the $[Ca^{2+}]_{cyt}$ response to agonist stimulation is increased, similar to the effect of inflammatory cytokine exposure. Importantly, exposure to TNFα or IL-13 blunted mitochondrial Ca^{2+} uptake during agonist stimulation, suggesting an uncoupling of the SR/ER and mitochondria and a reduction in mitochondrial buffering of $[Ca^{2+}]_{cyt}$. The effect of ER/SR stress on $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mito}$ in ASM has not been previously reported, but in other cell types it induces an increase in $[Ca^{2+}]_{cyt}$. Similarly, ablation of the Mfn2 gene increases basal $[Ca^{2+}]_{cyt}$ and reduces $[Ca^{2+}]_{mito}$, similar to the effect of inflammatory cytokine exposure in ASM.

Elevation of $[Ca^{2+}]_{mito}$ and excitation/energy coupling

Elevation of $[Ca^{2+}]_{mito}$ stimulates enzymes of the tricarboxylic acid (TCA) cycle, and thus, the downstream electron transport chain (Brookes et al. 2004; Denton 2009; Gunter and Sheu 2009; Glancy and Balaban 2012). Therefore, mitochondrial coupling with the ER/SR membrane to create a Ca^{2+} hotspot and cause mitochondrial Ca^{2+} uptake (increased $[Ca^{2+}]_{mito}$) is critical for matching the increased energy demands that result from cross-bridge recruitment and cycling via excitation–contraction coupling (triggered by transient elevation of $[Ca^{2+}]_{cyt}$) and increased mitochondrial ATP production (i.e., excitation–energy coupling) (Fig. 5) (Schaible et al. 2014). An abnormal increase or decrease in $[Ca^{2+}]_{mito}$ (relative to the transient $[Ca^{2+}]_{cyt}$ response) is associated with mitochondrial dysfunction and increased ROS generation (Malli et al. 2003; Brookes et al. 2004; Romagnoli et al. 2007; Dada and Sznajder 2011; Csordas et al. 2012; Dorn and Maack 2013).

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Cytokine-induced mitochondrial fragmentation

Apart from its role in ER/SR-mitochondrial coupling, Mfn2 is also essential in the fusion of mitochondrial filamentous networks within ASM. Mitochondria normally exhibit tubular, reticular, or networked morphology, which is regulated by dynamic remodeling, represented by a balance between fission vs. fusion that shifts in response to the environment (Chan 2006*a*, 2006*b*, 2012; Liesa et al. 2009; Youle and van der Bliek 2012). The balance between fission and fusion is now thought to be important for mitochondrial DNA stability, respiratory function, cell fate determination, and adaptation to cellular stress (Chan 2012). In mammalian cells, mitochondrial fusion involves 2 steps: fusion of the outer membrane, which is dependent on 2 GTPase proteins, mitofusin 1 (Mfn1) and 2 (Mfn2); and fusion of the inner membrane, which requires optic atrophy protein 1 (OPA1) (Fig. 9). The fusion of the outer mitochondrial membrane is initiated by dimerization of mitofusins and tethering of outer membranes of adjacent mitochondria. The fusion of the inner membranes of the mitochondria requires a motor-like process driven by OPA1 (Song et al. 2009; Palmer et al. 2011; Ranieri et al. 2013) Mitochondrial fission depends largely on dynamin related protein 1 (Drp1), which promotes fission by tethering to mitochondria at specific positions known as constriction sites and then forming multimeric spirals around mitochondria further constricting mitochondrial tubules leading to fission/fragmentation. In human cells, it has been reported that fission 1 protein (hFis1), present in the outer membrane, is involved in recruiting cytosolic Drp1 to constriction sites (Smirnova et al. 2001; James et al. 2003; Lee et al. 2004; Sheridan and Martin 2010). We recently reported that Mfn2 expression is reduced, whereas Drp1 is increased in hASM from patients with moderate asthma (Aravamudan et al. 2014). As a result, we also found that mitochondrial fragmentation is increased in asthmatic hASM. Recent evidence has suggested that Mfn2 and dynamic mitochondrial remodeling (balance between fusion and fission) are involved in pro-proliferative pathways (Liesa et al. 2009; Antico Arciuch et al. 2012).

Cytokine-induced mitochondrial fragmentation and cell proliferation in ASM

In the most fundamental sense, mitochondrial fragmentation and elongation is required for mitochondrial biogenesis to ensure that mitochondrial number is maintained subsequent to cell division (Antico Arciuch et al. 2012). Interestingly, studies have shown that mitochondrial biogenesis is enhanced in asthmatic ASM (Trian et al. 2007; Girodet et al. 2011); however, these studies did not examine mitochondrial network morphology. As a first step in mitochondrial biogenesis, fragmentation may reflect or induce an increased proliferative state of the cell. Indeed, inducing mitochondrial fragmentation leads to cell proliferation (Liesa et al. 2009; Antico Arciuch et al. 2012). Recent studies have suggested that in vascular smooth muscle, Mfn2 plays a regulatory role in cell division (Liesa et al. 2009; Antico Arciuch et al. 2012). In vascular smooth muscle, Mfn2 overexpression leads to cell growth arrest (Liesa et al. 2009). In addition, Mfn2 may inhibit the activation of pro-proliferative kinases such as extracellular signal-regulated kinase (ERK1/2) (Liesa et al. 2009). Importantly, many studies suggest that ERK1/2 activation plays a critical role in

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mediating ASM proliferation (Lee et al. 2001; Yu et al. 2013; Dragon et al. 2014;Movassagh et al. 2014).

ER/SR stress in ASM provides potential therapeutic targets for asthma

There is a growing amount of evidence to indicate that inflammation/ROS-induced ER/SR stress is a major contributor in the pathophysiology of various diseases, now including asthma. Recently, ER/SR stress markers have been identified in lungs from mouse models of airway diseases and chemical chaperones. Inhibition of ER/SR stress responses successfully reduced airway inflammation and airway hyper-reactivity observed in those models (Hoffman et al. 2013; Kim et al. 2013; Makhija et al. 2013). The results of our studies on hASM cells support a central role of inflammatory cytokine-induced ER/SR stress in both the enhanced contractile response of ASM (hyper-reactive state) and the increase in ASM cell proliferation (synthetic state) (Fig. 10). The functional impact of ER/SR stress in ASM cells is mediated in large part by the reduced expression of Mfn2. Thus, these results suggest potential therapeutic target in asthma that include: (*i*) ROS scavengers, (*ii*) chemical chaperones, and (*iii*) gene therapy to increase Mfn2 expression.

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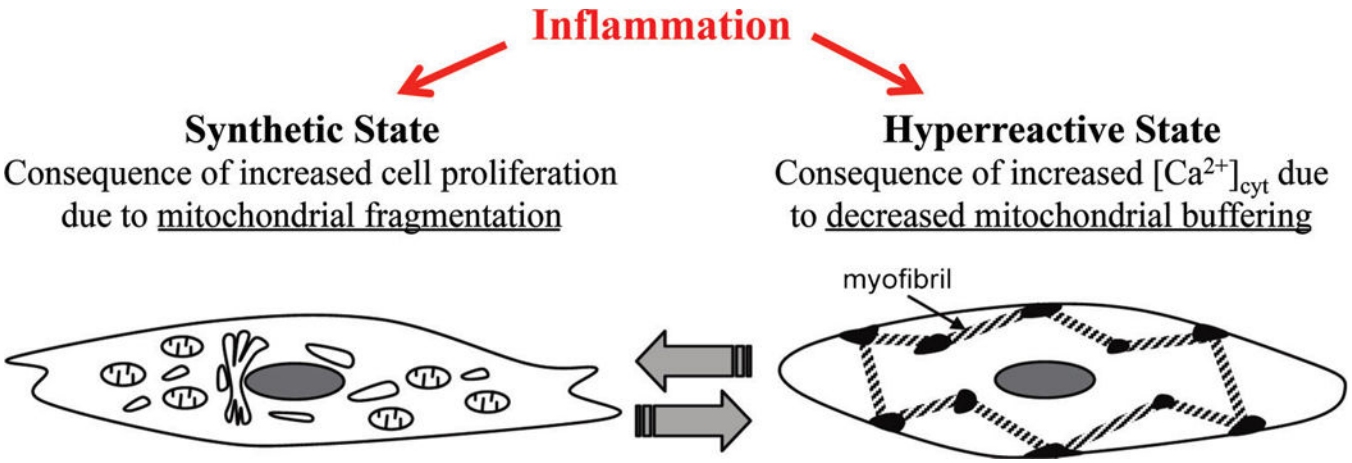

Fig. 1.

Inflammation plays a central role in asthma pathophysiology inducing both increased cell proliferation (synthetic state) and an enhanced contractile response to agonist stimulation (hyper-reactive state) in airway smooth muscle (ASM). We hypothesize that increased ASM cell proliferation is associated with decreased mitofusin-2 (Mfn2) expression and mitochondrial fragmentation, while ASM hyper-reactivity results from an increased $[Ca^{2+}]_{cyt}$ due to an uncoupling of mitochondria from the endoplasmic reticulum/sarcoplasmic reticulum membrane (decreased Mfn2 expression) and decreased mitochondrial Ca^{2+} buffering (reduced $[Ca^{2+}]_{mito}$ response).

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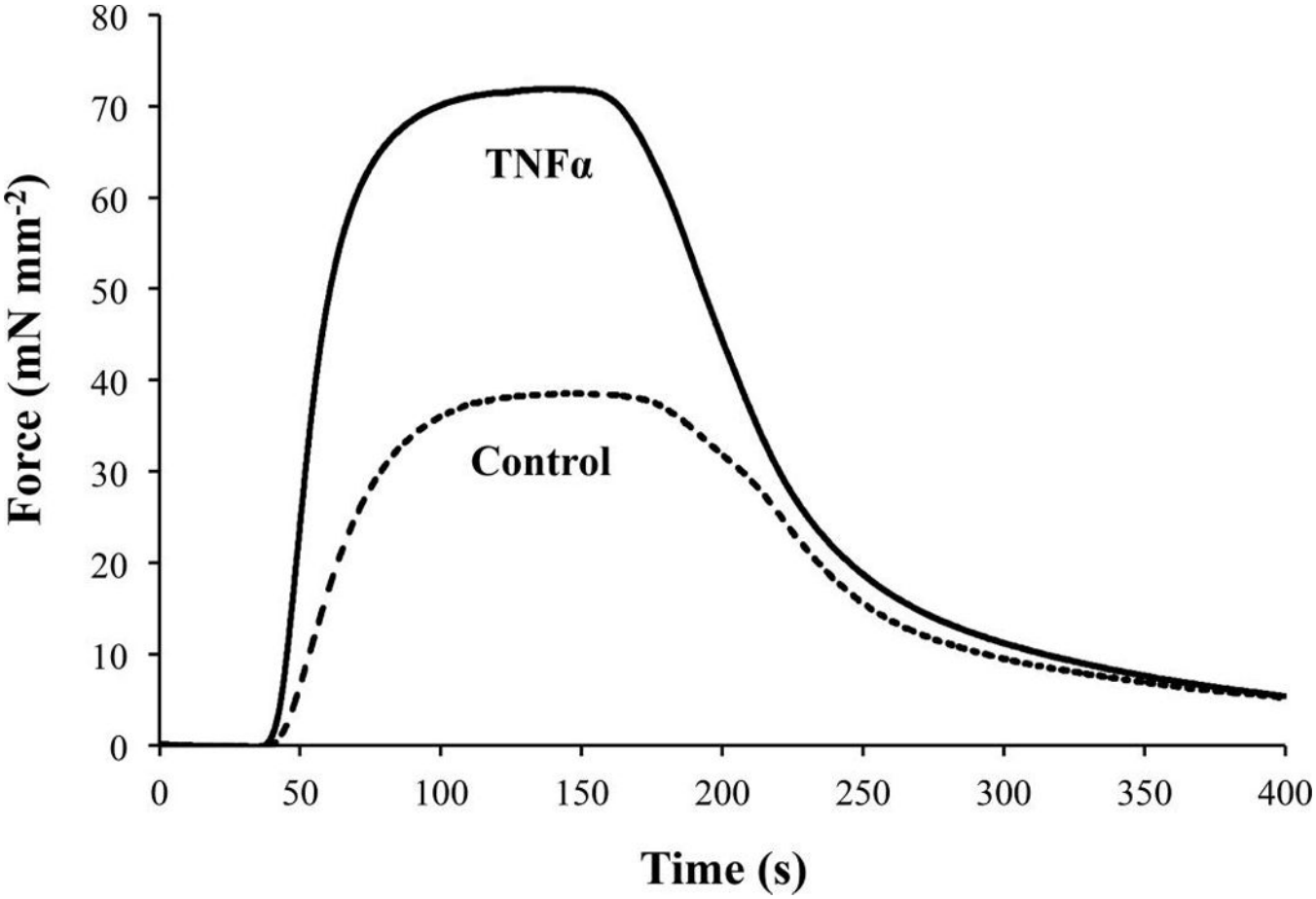

Fig. 2.

Exposure to tumor necrosis factor α (TNFα; 20 ng/mL for 24 h) increases the isometric force response of permeabilized (Triton X-100; 1%) porcine tracheal smooth muscle activated by 10 μmol·L^{-1} free Ca^{2+} (n = 4).

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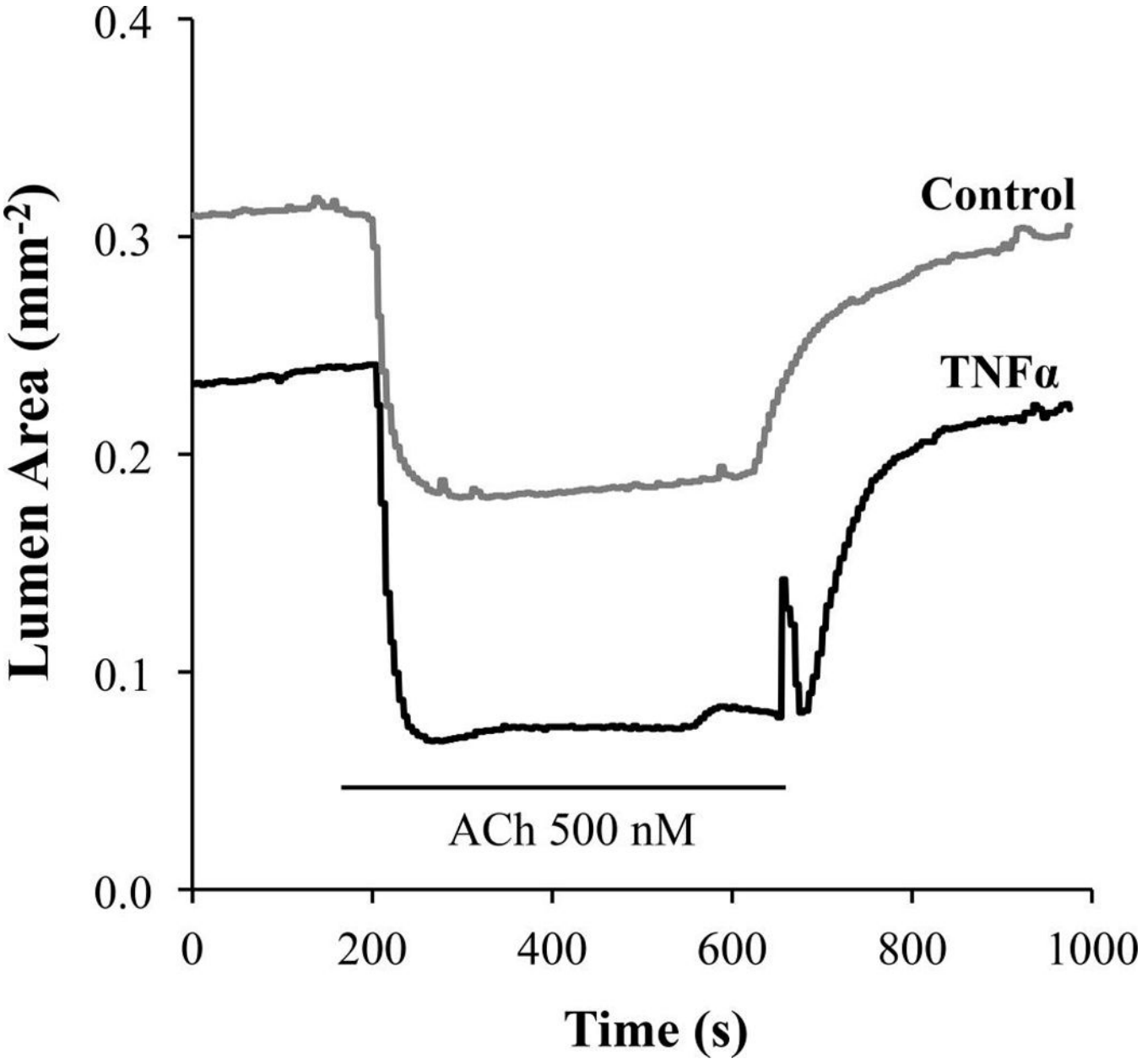

Fig. 3.

In a mouse lung slice, tumor necrosis factor α (TNFα) exposure (20 ng/mL for 12 h) increases constriction of small bronchioles induced by 500 nmol·L^{-1} acetylcholine (ACh) (*n* = 2 mice).

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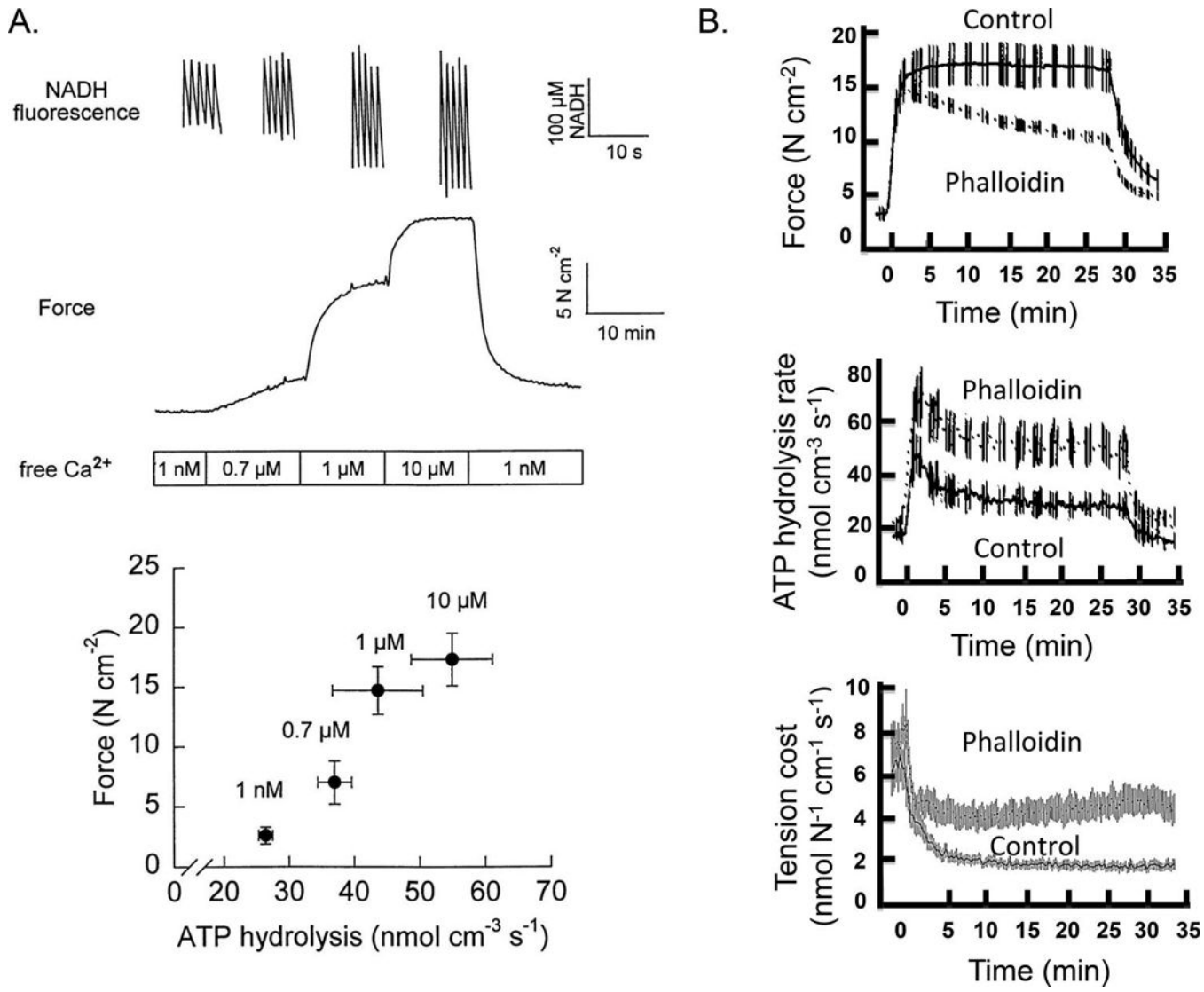

Fig. 4.

(A) Changes in isometric force and ATP hydrolysis rate (measured via extinction of NADH fluorescence) in Triton X-100 (0.1%)-permeabilized canine tracheal smooth muscle activated by various levels of free Ca^{2+} (1.0 nmol·L^{-1} to 10.0 µmol·L^{-1}). Note the linear relationship between isometric force and ATP hydrolysis rate. (B) Time course of changes in isometric force, ATP hydrolysis rate, and tension cost of permeabilized canine tracheal smooth muscle activated by 10 µmol·L^{-1} free Ca^{2+}. During normal isometric activation of airway smooth muscle (ASM), ATP hydrolysis rate is initially faster and then declines with time to a sustained level, even though isometric force is maintained. Tension cost (ATP consumption per unit force generation) of ASM activation also starts higher and then decreases with time. Phalloidin induced disruption of actin cytoskeletal remodeling results in force decline and blunted the time-dependent changes in ATP hydrolysis rate and tension cost. (Modified from Fig. 2 of Jones et al. 1999*b* and from Figs. 2 and 4 of Jones et al. 1999*a*).

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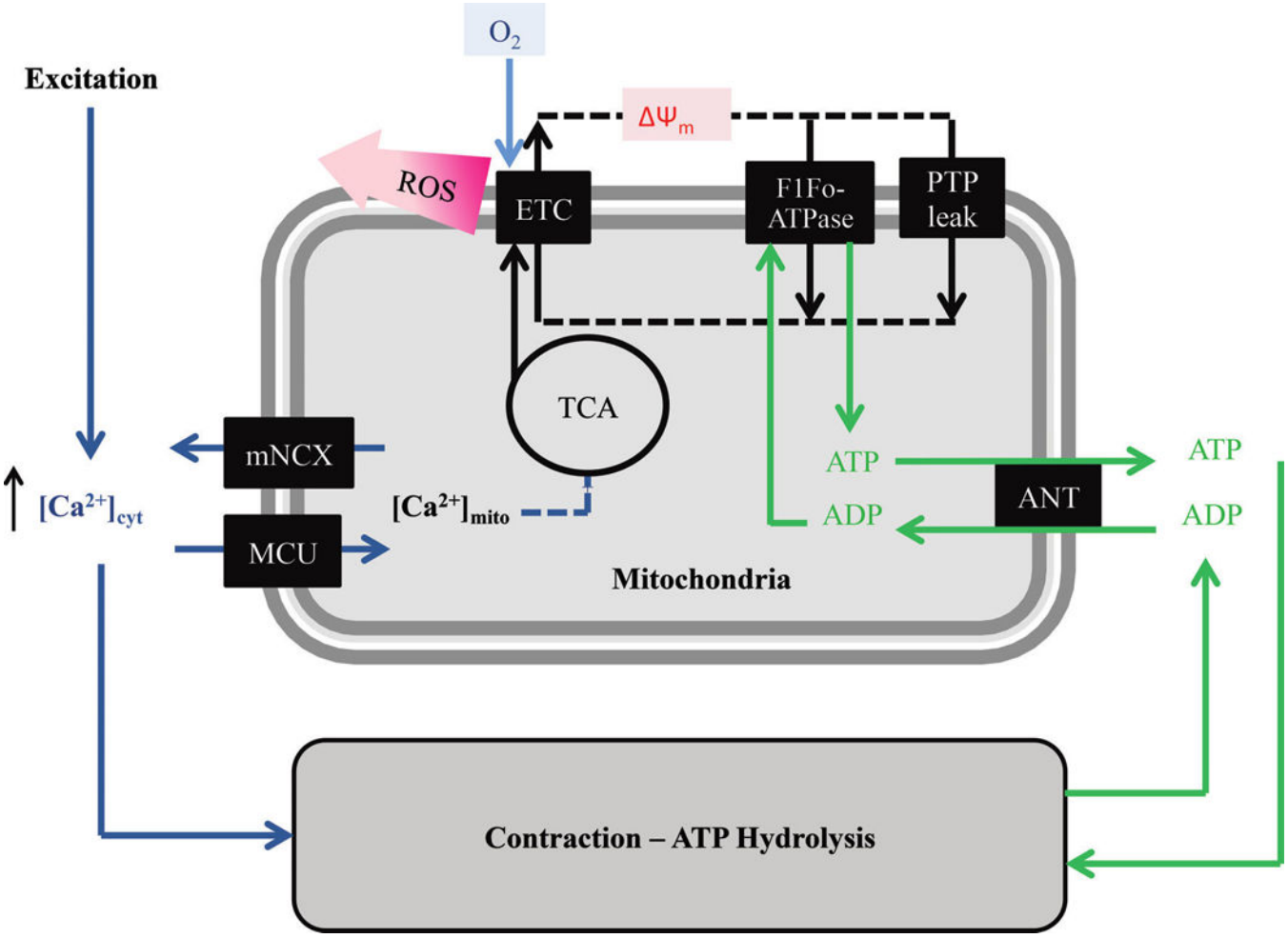

Fig. 5.

In airway smooth muscle cells, the agonist-induced transient increase of $[Ca^{2+}]_{cyt}$ is accompanied by activation of the MCU and a transient increase in $[Ca^{2+}]_{mito}$ thereby increasing activities of intra-mitochondrial dehydrogenases of the TCA cycle and the amount of NADH available to the electron transport chain. Mitochondrial O_2 consumption and ATP synthesis are also linked to cross-bridge cycling and ATP hydrolysis via changes in the ATP/ADP ratio and the ANT. A portion of the O_2 consumed in the ETC is incompletely reduced resulting in ROS. MCU, mitochondrial Ca^{2+} uniporter; TCA, tricarboxylic acid; ANT, adenine nucleotide transporter; ETC, electron transport chain; ROS, reactive oxidant species; mNCX, mitochondrial Na–Ca exchanger; F_1F_o, F-type ATPases; PTP, permeability transition pore.

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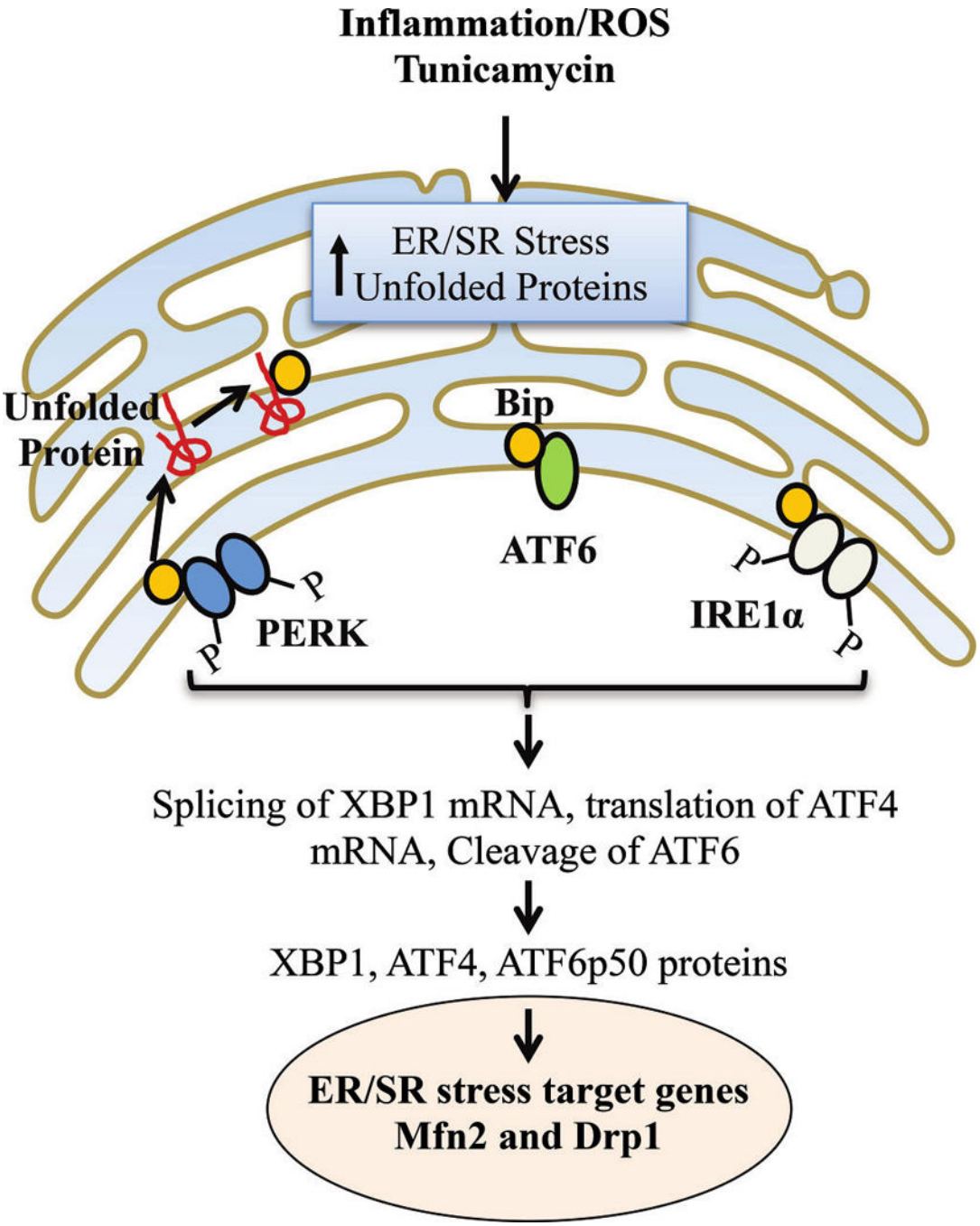

Fig. 6.

In hASM, inflammation, ROS, and (or) tunicamycin induce an accumulation of unfolded proteins and ER/SR stress (unfolded protein response). The molecular chaperone BiP (GRP78) is normally associated with PERK, ATF6, and IRE1α and maintains these proteins in an inactive state. As unfolded proteins accumulate in the ER/SR lumen, expression of BiP increases, and it dissociates from PERK, ATF6, and IRE1 leading to their activation and resulting in selective translation of the transcription factor ATF4; active transcription factor ATF6 (i.e., ATF6p50); and the active (spliced) XBP1 transcription factor. The IRE1, ATF6,

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and PERK pathways act to: (*i*) block protein translation, (*ii*) increase chaperone expression (e.g., calnexin), (*iii*) enhance SR-associated protein degradation pathways, and (*iv*) alter mitochondrial fusion (Mfn2) and fission (Drp1) protein expression. ROS, reactive oxidant species; hASM, human airway smooth muscle cells; ER/SR, endoplasmic reticulum/sarcoplasmic reticulum; BiP, binding immunoglobulin protein; PERK, protein kinase RNA-like endoplasmic reticulum kinase; ATF, active transcription factor; XBP1, X-box binding protein 1; IRE1, inositol-requiring enzyme 1; Mfn2, mitofusin-2; Drp1, dynamin related protein 1.

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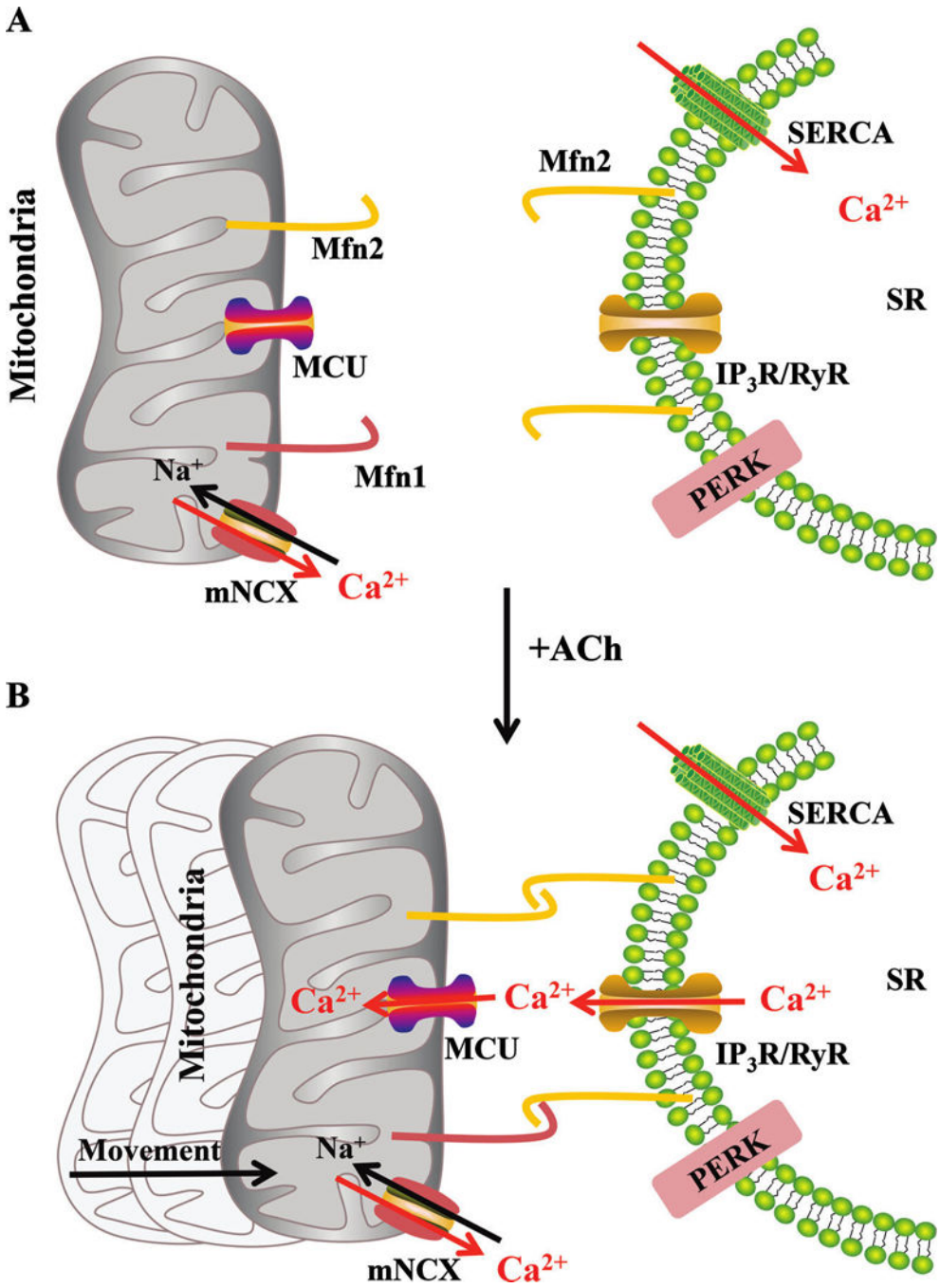

Fig. 7.

In hASM, agonist-induced elevations of $[Ca^{2+}]_{cyt}$ are associated with corresponding elevations in $[Ca^{2+}]_{mito}$, reflecting mitochondrial Ca^{2+} buffering via activation of the MCU that requires close proximity of mitochondria to the ER/SR membrane. Activation of MCU is only achieved within localized microdomains (i.e., "Ca^{2+} hot spots") in the vicinity of the IP_3R or RyR. This mitochondrial coupling to the ER/SR membrane requires mitochondrial movement and tethering mediated by dimerization of Mfn2s (homodimer) or Mfn2 and Mfn1 (hetrodimer). Subsequent to agonist activation, $[Ca^{2+}]_{mito}$ decreases via the mNCX.

Refilling of the ER/SR is achieved by SERCA. hASM, human airway smooth muscle cells; MCU, mitochondrial Ca^{2+} uniporter; IP_3R, inositol 1,4,5-triphosphate receptor; RyR, ryanodine receptor; ER/SR, endoplasmic reticulum/sarcoplasmic reticulum; mNCX, mitochondrial Na–Ca exchanger; SERCA, SR calcium transport ATPase.

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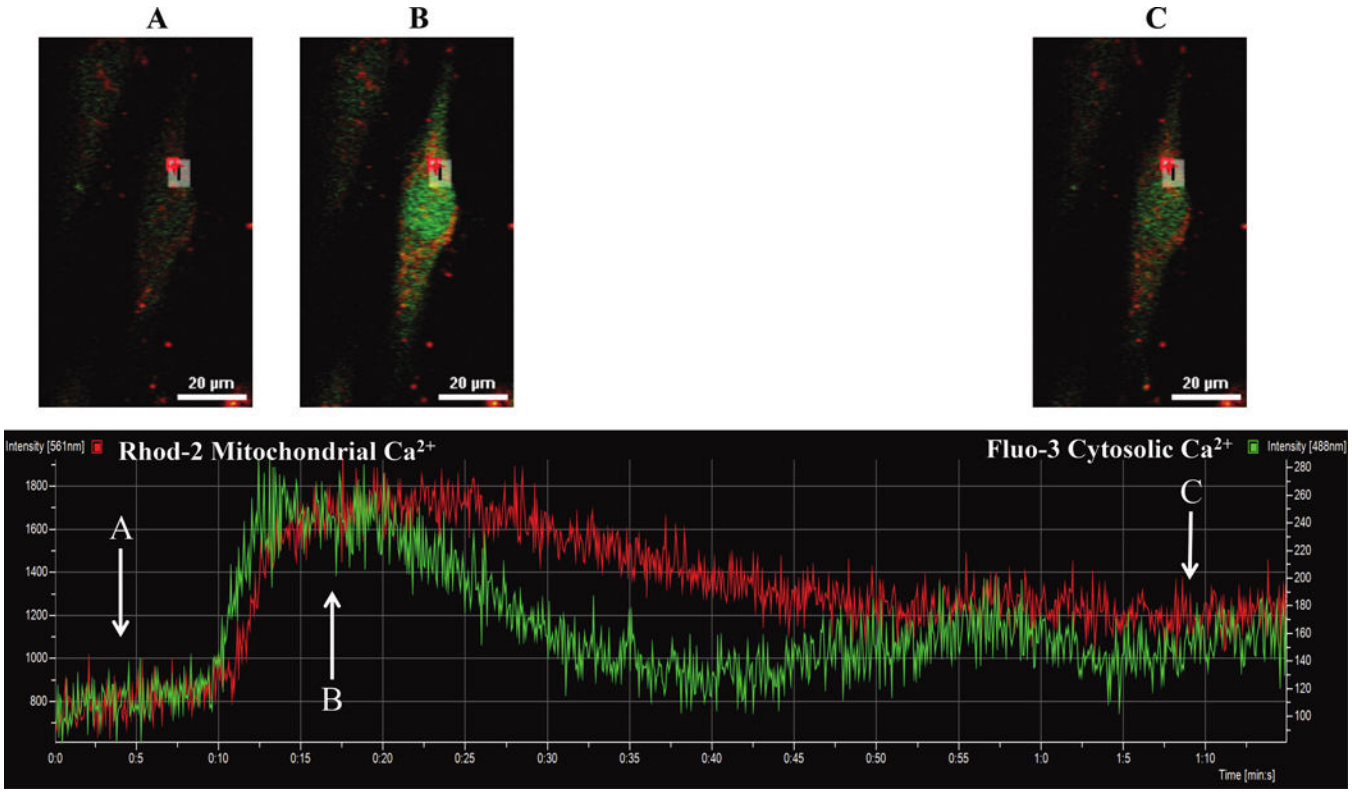

Fig. 8.

Human ASM cells were loaded with 2.5 µmol·L^{-1} fluo3-AM (green) and 2.5 µmol·L^{-1} rhod-2-AM (red) to measure changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mito}$, respectively (top: images at 3 different time points (A, B, and C) relative to ACh stimulation). Fluo3-AM and rhod-2-AM fluorescence increased in response to 1.0 µmol·L^{-1} ACh stimulation (bottom). Note that the transient $[Ca^{2+}]_{cyt}$ response slightly preceded the $[Ca^{2+}]_{mito}$ response, whereas the $[Ca^{2+}]_{mito}$ response was more prolonged. ASM, airway smooth muscle cells; ACh, acetylcholine.

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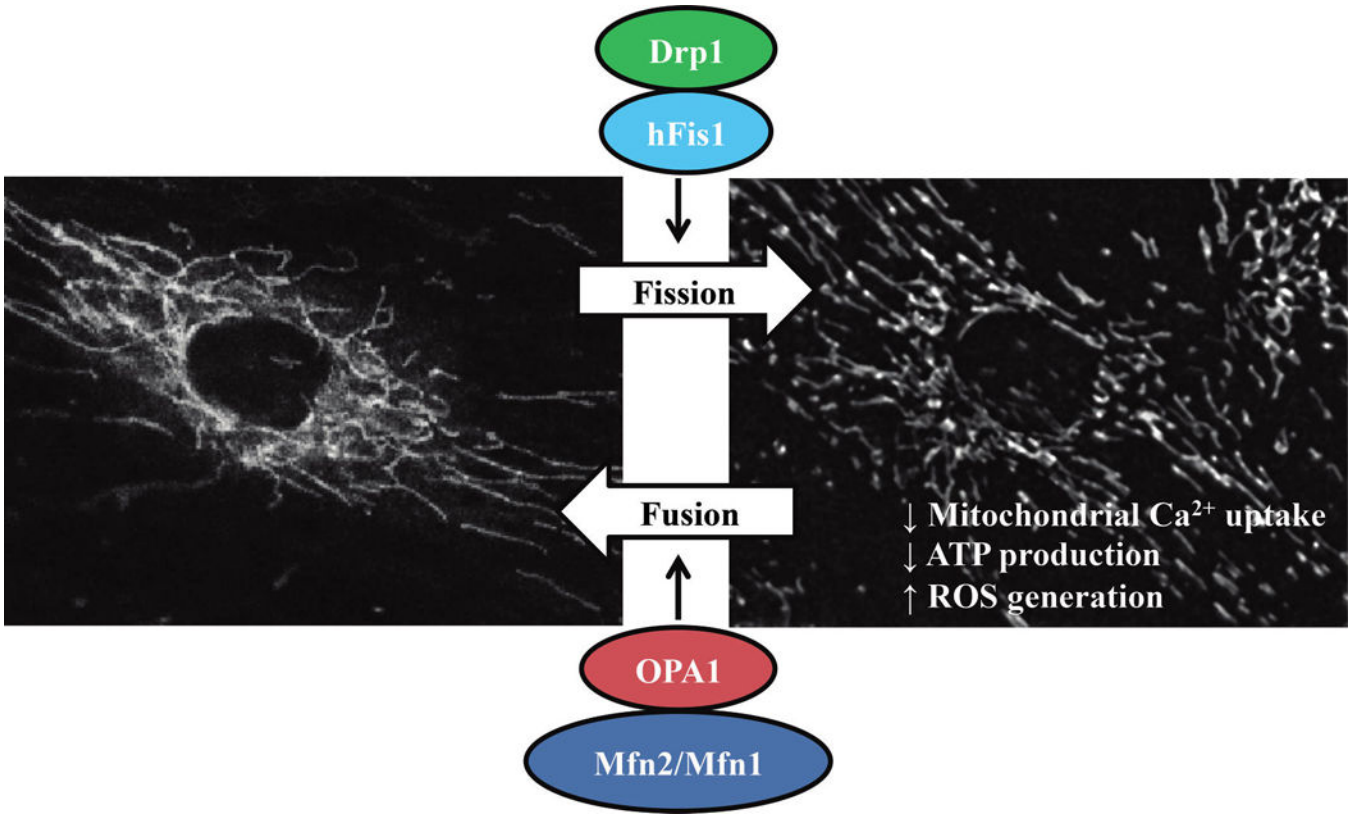

Fig. 9.

Human ASM cells were loaded with 500 nmol·L^{-1} MitoTraker Green AM to visualize the mitochondrial network. Control hASM cells displayed a filamentous/elongated mitochondrial network (fusion), which is promoted by Mfn1, Mfn2, and OPA1 proteins. In contrast, after 24 h exposure to TNFα (20 ng/mL for 12 h), mitochondria in hASM cells were more fragmented (fission). Mitochondria fragmentation (fission) is promoted by Drp1 and hFis1 proteins, and is associated with reduced mitochondrial Ca^{2+} uptake, ATP production, and increased ROS generation. ASM, airway smooth muscle; ROS, reactive oxidant species; Mfn, mitofusin; Drp1, dynamin related protein 1; OPA1, optic atrophy protein 1.

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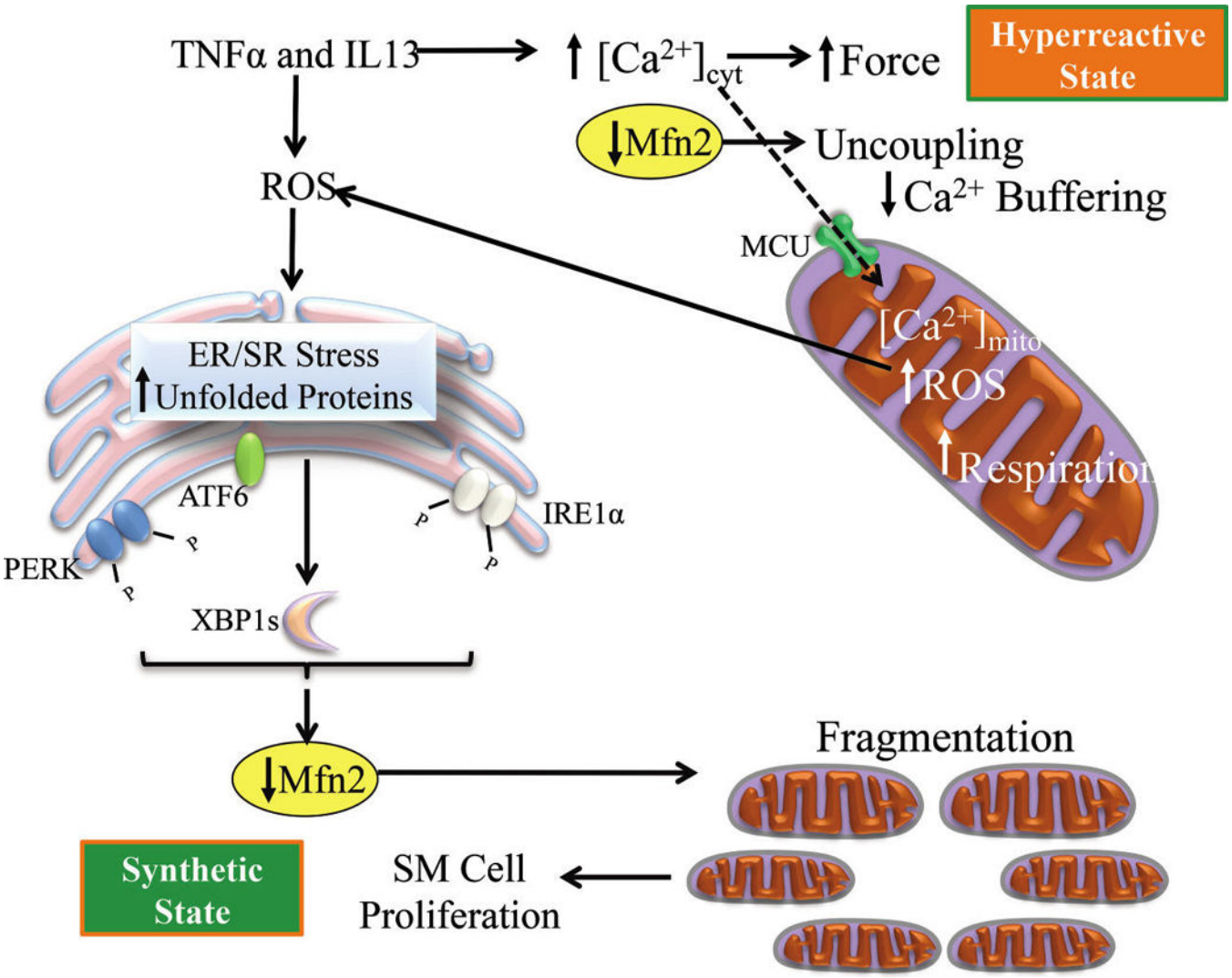

Fig. 10.

Inflammatory cytokines induce ROS generation in hASM that leads to ER/SR stress with downstream impact of reduced Mfn2 expression (increased Drp1), uncoupling of mitochondria to the ER/SR membrane, decreased $[Ca^{2+}]_{mito}$ buffering, which contributes to increased $[Ca^{2+}]_{cyt}$ and force responses to ACh stimulation (hyper-reactive state). A decrease in $[Ca^{2+}]_{mito}$ buffering also leads to mitochondrial dysfunction and a further increase in ROS generation. Reduced Mfn2 expression also leads to mitochondrial fragmentation (fission) and increased cell proliferation and remodeling (synthetic state). ROS, reactive oxidant species; hASM, human airway smooth muscle cells; ER/SR, endoplasmic reticulum/sarcoplasmic reticulum; ACh, acetycholine; Mfn2, mitofusin-2; Drp1, dynamin related protein 1.