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REVIEW
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Endoplasmic Reticulum Stress and Mitochondrial Function in Airway Smooth Muscle

*Philippe Delmotte and Gary C. Sieck**

Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN, United States

Inflammatory airway diseases such as asthma affect more than 300 million people worldwide. Inflammation triggers pathophysiology via such as tumor necrosis factor α (TNFα) and interleukins (e.g., IL-13). Hypercontraction of airway smooth muscle (ASM) and ASM cell proliferation are major contributors to the exaggerated airway narrowing that occurs during agonist stimulation. An emergent theme in this context is the role of inflammation-induced endoplasmic reticulum (ER) stress and altered mitochondrial function including an increase in the formation of reactive oxygen species (ROS). This may establish a vicious cycle as excess ROS generation leads to further ER stress. Yet, it is unclear whether inflammation-induced ROS is the major mechanism leading to ER stress or the consequence of ER stress. In various diseases, inflammation leads to an increase in mitochondrial fission (fragmentation), associated with reduced levels of mitochondrial fusion proteins, such as mitofusin 2 (Mfn2). Mitochondrial fragmentation may be a homeostatic response since it is generally coupled with mitochondrial biogenesis and mitochondrial volume density thereby reducing demand on individual mitochondrion. ER stress is triggered by the accumulation of unfolded proteins, which induces a homeostatic response to alter protein balance via effects on protein synthesis and degradation. In addition, the ER stress response promotes protein folding via increased expression of molecular chaperone proteins. Reduced Mfn2 and altered mitochondrial dynamics may not only be downstream to ER stress but also upstream such that a reduction in Mfn2 triggers further ER stress. In this review, we summarize the current understanding of the link between inflammation-induced ER stress and mitochondrial function and the role played in the pathophysiology of inflammatory airway diseases.

Keywords: mitofusin, IRE1, XBP1, asthma, inflammation

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***Correspondence:**
Gary C. Sieck
sieck.gary@mayo.edu

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INTRODUCTION

Inflammation triggers asthma pathophysiology via pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 13 (IL-13). Two hallmarks of asthma are human airway smooth muscle (hASM) hypercontractility and cell proliferation (James, 2005; Joubert and Hamid, 2005; Black et al., 2012; Prakash, 2013, 2016; Wright et al., 2013a,b; Delmotte and Sieck, 2015). It is likely that with asthma, hASM exists in both hyper-contractile and proliferative states, thus contributing to a thicker, more contractile airway. An emergent theme in this context is the role of inflammation-induced endoplasmic reticulum (ER) stress and mitochondria. Previously,

we showed that cytokine exposure increases agonist-induced hASM force and ATP consumption due to an increase in contractile protein expression (Dogan et al., 2017). Initially, the increase in hASM ATP demand is met by increased mitochondrial O_2 consumption and ATP production, but at the expense of reactive oxygen species (ROS) formation and oxidative stress. There is increasing evidence that mitochondria and the ER, although structurally separate organelles, are functionally interdependent units, which must establish links for normal cellular function, including $[Ca^{2+}]_{cyt}$ regulation, energy production and cell proliferation (Hajnoczky et al., 2000; Franzini-Armstrong, 2007; Romagnoli et al., 2007; Liesa et al., 2009; Kornmann and Walter, 2010; Antico Arciuch et al., 2012; Glancy and Balaban, 2012; Dorn and Maack, 2013; Kornmann, 2013; Raturi and Simmen, 2013; van Vliet et al., 2014; Filadi et al., 2017). These links are established through specialized ER-mitochondria encounter structures (ERMES) comprising both ER and mitochondrial transmembrane proteins that provide a tethering force between the two organelles to ensure proximity and communication (Franzini-Armstrong, 2007; Kornmann and Walter, 2010; Dorn and Maack, 2013; Kornmann, 2013; Raturi and Simmen, 2013; van Vliet et al., 2014; Filadi et al., 2017). Mitofusin-2 (Mfn2) is an ERMES component that serves to tether mitochondria to the ER. Mfn2 also serves to fuse mitochondria, which together with other fusion proteins [e.g., Mfn1, optic atrophy 1 (Opa1)] elongate mitochondria making them more filamentous, whereas fission proteins such as dynamin related protein 1 (Drp1) and fission 1 protein (Fis1) act to fragment mitochondria. Together these fusion/fission proteins act to dynamically remodel mitochondria under a variety of conditions (Smirnova et al., 2001; James et al., 2003; Lee et al., 2004; Song et al., 2009; Sheridan and Martin, 2010; Palmer et al., 2011; Ranieri et al., 2013). The tethering of mitochondria to the ER allows mitochondrial proximity to ER Ca^{2+} release sites representing a microdomain of higher $[Ca^{2+}]_{cyt}$ ("hotspot" $>$ 2 μM) that is essential for mitochondrial Ca^{2+} influx [by activating the mitochondrial Ca^{2+} uniporter (MCU)] (Raffaello et al., 2012). In the absence of mitochondrial Ca^{2+} buffering, the transient $[Ca^{2+}]_{cyt}$ response to agonist stimulation is elevated, thereby enhancing hASM force generation. This review will discuss the link between ER stress, Mfn2 expression, mitochondrial tethering to the ER, mitochondrial Ca^{2+} influx, and mitochondrial function in the context of airway inflammation and potential consequences on ASM hyper-contractile and proliferative states.

INFLAMMATION AND ER STRESS IN HUMAN ASM

One consequence of inflammation is the unfolding of proteins that accumulate in the lumen of the ER, exposing binding sites for the chaperone protein, binding immunoglobulin protein (BiP). With an accumulation of unfolded proteins, BiP dissociates from three proteins localized at the ER membrane resulting in their activation. The resulting physiological response referred as ER stress or unfolded protein response attempts to restore normal ER function by increasing chaperones proteins expression, halting protein translation and activating protein degradation (Yoshida et al., 2001; Bravo et al., 2012; Garg et al., 2012; Verfaillie et al., 2012; Hasnain et al., 2013; Sano and Reed, 2013; Vannuvel et al., 2013; Delmotte and Sieck, 2015; Kim and Lee, 2015; Zeeshan et al., 2016; Jeong et al., 2017; Navid and Colbert, 2017; Shanahan and Furmanik, 2017; Morris et al., 2018). These three ER stress protein markers involved in this signaling cascade are: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1α, also called serine/threonine-protein kinase/endoribonuclease IRE1α) (**Figure 1**) (Hai et al., 1989; Nikawa and Yamashita, 1992; Cox et al., 1993; Harding et al., 1999; Li et al., 2000). Phosphorylation of IRE1α (pIRE1α) catalyzes the alternative splicing of XBP1 mRNA (XBP1s) and expression of the active XBP1s transcription factor. Generally, the pIRE1α/XBP1s pathway is associated with upregulation of chaperone protein expression that serves to promote protein refolding and restore ER homeostasis. The RNAse activity of IRE1α is also involved in the regulation of mRNAs through a mechanism called regulated IRE1-dependent decay of mRNA (RIDD) (Hollien and Weissman, 2006). Interestingly, IRE1α could also cleave several pre-miRNAs which could potentially regulate a number of mRNA targets (Upton et al., 2012). As a result, RIDD and therefore, ER stress could affect directly and indirectly a large number of mRNA targets. ATF6 translocates to the Golgi apparatus and is cleaved first by site 1 protease (S1P) and second by site 2 protease (S2P) leading to an active ATF6 transcription factor. As for the pIRE1α/XBP1s pathway, the ATF6 pathway is usually associated with upregulation of chaperone protein expression but also with autophagy, lipid synthesis and endoplasmic-reticulum-associated protein degradation (ERAD) (Yoshida et al., 2001; Bravo et al., 2012; Garg et al., 2012;

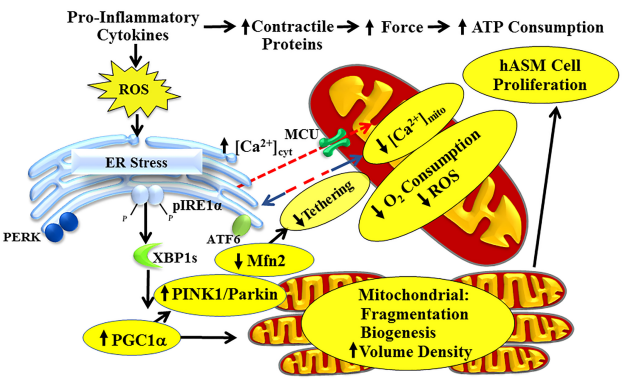

FIGURE 1 | Pro-inflammatory cytokines activate the pIRE1α/XBP1s ER stress pathway in hASM, leading to increased PGC1α and reduced Mfn2 expression. Reduced Mfn2 disrupts tethering of mitochondria to the ER, thereby decreasing mitochondrial Ca^{2+} influx and reducing O_2 consumption of individual mitochondrion. Increased PGC1α induces mitochondrial biogenesis and increases mitochondrial volume density to meet increased ATP demand. Cytokines also increase hASM force and ATP consumption by increasing contractile protein expression, thereby increasing energetic demand of individual hASM cells. This is mitigated by inducing hASM cell proliferation.

Verfaillie et al., 2012; Hasnain et al., 2013; Sano and Reed, 2013; Vannuvel et al., 2013; Delmotte and Sieck, 2015; Kim and Lee, 2015; Zeeshan et al., 2016; Jeong et al., 2017; Navid and Colbert, 2017; Shanahan and Furmanik, 2017; Morris et al., 2018). The role of ATF6 in the upregulation of XBP1 and the transcription factor C/EBP homologous protein (CHOP, ER stress-induced apoptosis) is also well documented and reviewed in Hu et al. (2018). Finally, PERK phosphorylates the translation-initiator factor eukaryotic initiation factor 2 (eIF2α), resulting in the translation of activating transcription factor 4 (ATF4). ATF4 is involved in the upregulation of CHOP, ERAD and mitophagy pathways (Adolph et al., 2012; Hasnain et al., 2012, 2013; Dromparis et al., 2013; Kim and Lee, 2015; Zeeshan et al., 2016; Jeong et al., 2017; Navid and Colbert, 2017; Shanahan and Furmanik, 2017; Hu et al., 2018; Morris et al., 2018). In cell types other than hASM, inflammation has been shown to induce ER stress (Adolph et al., 2012; Garg et al., 2012; Hasnain et al., 2012, 2013; Baban et al., 2013; Martino et al., 2013). These studies also demonstrated that the ER stress response is highly variable depending on cell type, species and experimental condition, which subsequently leads to various downstream physiological effects. Inflammation-induced ER stress is most likely a consequence of increased ROS generation (Adolph et al., 2012; Garg et al., 2012; Hasnain et al., 2012, 2013; Baban et al., 2013; Martino et al., 2013), although it is unclear whether ROS is the only mechanism involved. In a recent study, we showed that, TNFα selectively activates the pIRE1α/XBP1s in non-asthmatic hASM cells (Yap et al., 2019). Whether cytokines other than TNFα also selectively activate the pIRE1α/XBP1s pathway is not known. Interestingly, TNFα increases superoxide formation in hASM and Tempol, a superoxide scavenger, reduces the effect of TNFα on the activation of pIRE1α/XBP1s pathway (Yap et al., 2019). To date no other study has explored whether inflammation induces ER stress in hASM and whether an ER stress response in hASM cells from asthmatic patients exists and/or is affected by inflammation. A few studies suggest that the ER stress response is exaggerated in airway epithelial cells or immune cells in the context of asthma (Kim and Lee, 2015; Jeong et al., 2017; Pathinayake et al., 2018). In a mouse model of asthma, chemical chaperones have been used to reduce the ER stress response and attenuate airway hyperresponsiveness (Makhija et al., 2014; Miller et al., 2014; Kim and Lee, 2015; Siddesha et al., 2016).

Mfn2 AND ER STRESS RESPONSE

In cells other than hASM, the relationship between the ER stress response and mitochondria has gain considerable interest. These previous studies have suggested that Mfn2 and altered mitochondrial dynamics are upstream to ER stress such that a reduction in Mfn2 triggers ER stress (Munoz and Zorzano, 2011; Ngoh et al., 2012; Schneeberger et al., 2013; Bhandari et al., 2015). In a recent study, we suggested that a reduction in Mfn2 in hASM cells is downstream to ER stress (Yap et al., 2019), creating the possibility of a vicious cycle with reduced Mfn2 expression and altered mitochondrial function at the center. Currently, the link between ER stress and downstream regulation of Mfn2 expression has been largely unexplored. A limited number of studies have examined specific downstream targets of activation of the pIRE1α/XBP1s pathway (Calfon et al., 2002; Fonseca et al., 2005; Lipson et al., 2006, 2008; Zeng et al., 2009), but none of these studies have examined effects on Mfn2 expression. As mentioned above, TNFα selectively activates the pIRE1α/XBP1s pathway and reduces Mfn2 expression (Yap et al., 2019), but how IRE1α phosphorylation and XBP1 mRNA splicing affects Mfn2 expression has not been examined in any cell type. Potential targets of interest include peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), mitophagy-related proteins PTEN-induced kinase 1 (PINK1) and Parkin, and miRNAs (**Figure 1**). Several studies found that XBP1s increases expression of PGC1α (Arensdorf et al., 2013; Cheang et al., 2017). Interestingly, PGC1α activates the PINK1/Parkin mitophagy pathway, which is involved in ubiquitination of Mfn2 (Chen and Dorn, 2013; Basso et al., 2018; McLelland et al., 2018). Similarly, a growing list of miRNA has been implicated in the downregulation of Mfn2 but it is not clear if they are expressed in hASM and whether XBP1 is involved in their regulation (Kuhn et al., 2010; Dileepan et al., 2016; Purohit et al., 2019). Previous studies have also shown that XBP1 induces several miRNA but their potential effect on Mfn2 has not been explored and again it's not known if these miRNA are expressed in hASM (Kuhn et al., 2010; Dileepan et al., 2016; Purohit et al., 2019). The PERK and ATF6 pathway have also been suggested to affect Mfn2 expression, either through PGC1α, mitophagy or ERAD pathways (Morris et al., 2018). It is not known if cytokines other than TNFα activate the PERK and ATF6 pathway in hASM and whether they are activated or amplified in asthmatic hASM. Conversely, the effect of Mfn2 knockdown on IRE1α phosphorylation and XBP1 mRNA splicing has only been examined by four studies – two in neurons, and one each in embryonic fibroblasts and Drosophila (Ngoh et al., 2012; Munoz et al., 2013; Schneeberger et al., 2013; Bhandari et al., 2015).

Mfn2 AND DYNAMIC REMODELING OF MITOCHONDRIA

In hASM, mitochondria are tubular or filamentous but this mitochondria morphology is highly dynamic with mitochondria constantly fusing (fusion) or breaking (fission) from one another. Mitochondria morphology is therefore, the result of this balance between fusion vs. fission (Chen and Chan, 2005; Chan, 2006, 2012; Liesa et al., 2009; Youle and van der Bliek, 2012). This dynamic remodeling is thought to be essential for mitochondrial DNA stability, respiratory function, and to adapt cellular stress resulting from ROS formation (Chan, 2012). Mitochondrial fusion involves the GTPases Mfn1 and/or Mfn2 responsible for the fusion of the outer membrane, and optic atrophy protein 1 (OPA1) for the fusion of the mitochondrial inner membrane (**Figure 2**). Mfn1 is located only at the mitochondrial outer membrane whereas Mfn2 is localized both at the mitochondrial membrane and in the cytosol. The dimerization of Mfn2 (Mfn2/Mfn2) and/or Mfn1 (Mfn1/Mfn2) tethers outer membranes of neighboring mitochondria (Song et al., 2009;

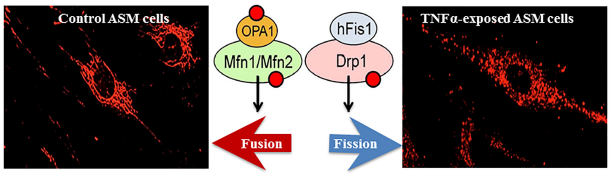

FIGURE 2 | In hASM, TNFα increases mitochondrial fragmentation (fission, visualized using MitoTracker Red); an effect mediated through a reduced expression of fusion proteins (Mfn2, Mfn1, and Opa1) and an increased expression of fission proteins (Drp1 and hFis1).

Palmer et al., 2011; Ranieri et al., 2013). Mitochondrial fission depends on the recruitment of cytosolic Drp1 by Fis1 to specific positions around mitochondria and known as constriction sites ultimately leading to fragmentation/fission of the mitochondria (Smirnova et al., 2001; James et al., 2003; Lee et al., 2004; Sheridan and Martin, 2010). The extent of fusion or fission of mitochondria can be quantified using morphological parameters such as form factor (perimeter2/4π×area) and/or aspect ratio (ratio of long and short axis) (Koopman et al., 2005a,b, 2006). We previously reported that Mfn2 expression was reduced in asthmatic hASM, and that this was correlated with an increase in mitochondria fragmentation (Aravamudan et al., 2014). A similar increase in mitochondrial fragmentation in hASM cells was observed after siRNA knockdown of Mfn2 (Aravamudan et al., 2017). In a recent study, we also found that TNFα reduces Mfn2 expression in hASM cells (Yap et al., 2019). As mentioned before, the relation between ER stress and Mfn2 expression and mitochondria fragmentation has been suggested but has not been clearly established.

ROLE OF Mfn2 IN TETHERING MITOCHONDRIA TO ER

There is converging evidence in other cell types that Mfn2 plays an essential role in tethering mitochondria to the ER (Hajnoczky et al., 2002; Patergnani et al., 2011; Raturi and Simmen, 2013; van Vliet et al., 2014; Filadi et al., 2017). Mfn2 located at the ER membrane can dimerize with Mfn2 (Mfn2/Mfn2) and/or Mfn1 (Mfn1/Mfn2) located at the mitochondrial membrane to tether mitochondria to the ER. In hASM cells, a transient $[Ca^{2+}]_{cyt}$ response induced by 1 μM ACh stimulation is accompanied by a transient $[Ca^{2+}]_{mito}$ response (Delmotte et al., 2012; Delmotte and Sieck, 2015). The transient $[Ca^{2+}]_{mito}$ response is blunted by inhibiting the MCU using Ru360. Mitochondrial Ca^{2+} influx via the MCU (Baughman et al., 2011; De Stefani et al., 2011) is only activated by microdomains of higher $[Ca^{2+}]_{cyt}$ ("hotspots" > 2 μM) (Gunter et al., 2000; Gunter and Gunter, 2001; Rizzuto and Pozzan, 2006; Gunter and Sheu, 2009; Rizzuto et al., 2009; Giacomello et al., 2010), which exceed the normal global transient $[Ca^{2+}]_{cyt}$ response to agonist stimulation in hASM (~500–600 nM) (Pabelick et al., 1999; Sieck et al., 2008; Sathish et al., 2009, 2011; Delmotte et al., 2012). Higher levels of $[Ca^{2+}]_{cyt}$ do occur after 24-h TNFα exposure in response to muscarinic stimulation (Delmotte et al., 2012; Delmotte and Sieck, 2015; Dogan et al., 2017; Sieck et al., 2019), but are still well below levels required to activate the MCU (Gunter et al., 2000; Gunter and Gunter, 2001; Rizzuto and Pozzan, 2006; Gunter and Sheu, 2009; Rizzuto et al., 2009; Giacomello et al., 2010). However, much higher levels of $[Ca^{2+}]_{cyt}$ ("hotspots") are observed in regions in close proximity to the ER Ca^{2+} efflux channels (IP_3 and RyR). Thus, during agonist stimulation, mitochondria must be tethered to the ER in order to establish close proximity of mitochondria to $[Ca^{2+}]_{cyt}$ "hotspots" for mitochondrial Ca^{2+} influx. We previously showed that TNFα disrupts mitochondrial proximity to the ER in hASM cells (Delmotte et al., 2017), but this study only suggests the potential involvement of reduced Mfn2 expression in hASM. Further studies will be necessary to provide direct evidence for the involvement of Mfn2. In hASM cells, it has not been established that Mfn2 is essential for tethering mitochondria to the ER, and thus, for establishing proximity of mitochondria to the ER and microdomains of higher $[Ca^{2+}]_{cyt}$ ("hotspots" > 2 μM). Such interactions are cell and context specific, so establishing the role of Mfn2 in hASM is critical. The potential effect of ER stress on microdomains of higher $[Ca^{2+}]_{cyt}$, and mitochondrial Ca^{2+} influx is likewise not clearly established.

EXCITATION-ENERGY COUPLING VIA MITOCHONDRIAL Ca^{2+} INFLUX

Based on biochemical studies, it is well known that mitochondrial production of ATP (oxidative phosphorylation) depends on dehydrogenase enzyme activities of the tricarboxylic acid (TCA) cycle (or citric acid cycle). Some of these dehydrogenase enzymes [i.e., pyruvate dehydrogenase (PDH), NAD-isocitrate dehydrogenase (ICDH), and oxoglutarate dehydrogenase (OGDH)] are Ca^{2+} dependent (Rizzuto et al., 2000; Parekh, 2003; Franzini-Armstrong, 2007; Maack and O'Rourke, 2007; Romagnoli et al., 2007). Additionally, an increase in $[Ca^{2+}]_{cyt}$ stimulates mitochondrial shuttle systems such as the glycerol phosphate shuttle and the aspartate/glutamate transporters resulting in an increase in NADH levels in the mitochondria (Palmieri et al., 2001; Denton, 2009). Thus, mitochondrial Ca^{2+} influx during transient elevation of $[Ca^{2+}]_{cyt}$ stimulates dehydrogenase enzyme activities within the TCA cycle and increases, O_2 consumption, electron transport chain (ETC) flux and ATP production – excitation-energy coupling (**Figure 3**). Conversely, it is well known that increased ATP consumption leads to transport of ADP into mitochondria via the adenosine nucleotide transporter (ANT), which stimulates ATP synthase (complex V) activity to match ATP production with ATP consumption (**Figure 3**). Together, these two energy sensing pathways form a normal homeostatic mechanism for excitation-energy coupling in a variety of cell types including hASM. Pathophysiology and mitochondrial dysfunction involve disruptions in these mitochondrial energy-sensing/signaling pathways. As mentioned, most of these studies involved biochemical studies and in some cases isolated mitochondria. While they are critical in our understanding of mitochondrial

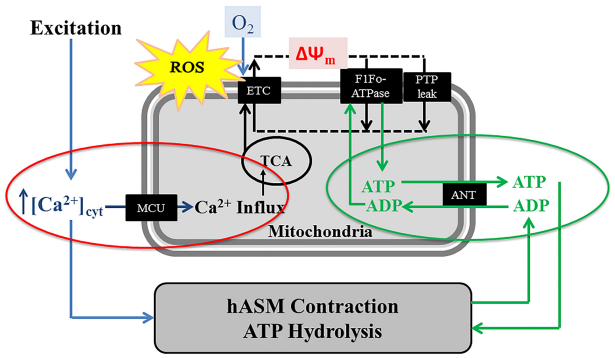

FIGURE 3 | In hASM cells, the agonist-induced transient increase of $[Ca^{2+}]_{cyt}$ (excitation) is accompanied by activation of the mitochondrial Ca^{2+} uniporter (MCU) and a transient increase in mitochondrial Ca^{2+} influx, thereby increasing activities of mitochondrial dehydrogenases of the TCA cycle and the electron transport chain (ETC). Mitochondrial O_2 consumption and ATP synthesis are also linked ATP hydrolysis (and therefore, hASM force) via changes in the ATP/ADP ratio and the adenine nucleotide transporter (ANT). A portion of the O_2 consumed in the ETC results in ROS formation.

function, there's a considerable need for new tools allowing to studies these mechanisms within single mitochondrion in live cells and tissues. A few imaging and/or molecular tools to measure mitochondrial membrane potential $\Delta\Psi_m$, succinate dehydrogenase (SDH) activity (Sieck et al., 1986, 1989, 1995, 1996), ATP consumption (Jones et al., 1999a,b; Dogan et al., 2017), ATP/ADP (Berg et al., 2009), and NAD/NADH ratio (Hung et al., 2011; Cohen et al., 2018) have been developed for use in live cells or tissue but have never been used in hASM.

ROLE OF ER STRESS IN MITOCHONDRIAL BIOGENESIS AND INCREASED MITOCHONDRIAL VOLUME DENSITY

A few studies reported that mitochondrial biogenesis in asthmatic hASM is increased but the mechanisms mediating this mitochondrial biogenesis were not explored (Trian et al., 2007; Girodet et al., 2011). These studies also did not examine mitochondria morphology and mitochondrial fragmentation/fission. An increase in mitochondrial volume density is an alternative mechanism to increase ATP production to meet increased ATP demand in hASM after exposure to pro-inflammatory cytokines. In this case, O_2 consumption in individual mitochondrion can be reduced to minimize formation of ROS. Recent evidence also suggests that reduced Mfn2 and mitochondrial fragmentation is required for mitochondrial biogenesis (Antico Arciuch et al., 2012; Peng et al., 2017), supporting our hypothesis that increased PGC1α and reduced Mfn2 are a coordinated homeostatic response to cytokine-induced activation of the pIRE1α/XBP1s ER stress pathway.

CYTOKINE EXPOSURE INCREASES ROS GENERATION IN hASM

A number of studies have reported that ROS generation increases in asthmatic patients (Katsumata et al., 1990; Comhair and Erzurum, 2010; Zuo et al., 2013) which has the potential to triggers ER stress in hASM. We recently showed exposure of non-asthmatic hASM to TNFα progressively increases superoxide anion formation (Yap et al., 2019). We also found that incubating hASM cells with Tempol (superoxide anion scavenger) mitigated the effects of TNFα in inducing ER stress as well as the reduction in Mfn2 (Yap et al., 2019). It is possible that an increase in ROS generation is triggered, in part by increased ATP consumption and mitochondrial O_2 consumption. Acute activation of the pIRE1α/XBP1s ER stress pathway leads to a transient reduction in Mfn2 thereby decreasing: (1) mitochondrial tethering to the ER (**Figure 1**); (2) mitochondrial Ca^{2+} influx (**Figures 1**, **3**); (3) TCA cycle dehydrogenase enzyme activity (**Figures 1**, **3**); (4) O_2 consumption (**Figures 1**, **3**); and as a result, (5) ROS formation (**Figures 1**, **3**). Without this homeostatic break on mitochondrial O_2 consumption, the increase in hASM force and ATP consumption induced by pro-inflammatory cytokines will increase ROS formation and further exacerbating ER stress.

CYTOKINE EXPOSURE INCREASES hASM FORCE, ATP CONSUMPTION AND TENSION COST

In previous studies, we showed that 24-h exposure of hASM cells to TNFα increases both $[Ca^{2+}]_{cyt}$ and force responses to 1 μM muscarinic (ACh) stimulation (White et al., 2006; Sathish et al., 2009, 2011; Delmotte et al., 2012; Jia et al., 2013; Delmotte and Sieck, 2015; Dogan et al., 2017; Sieck et al., 2019). However, hASM sensitivity to muscarinic stimulation is also increased after TNFα, which largely accounts for the enhanced $[Ca^{2+}]_{cyt}$ response, but not the force response (Sieck et al., 2019). In recent studies, we found that the increase in ASM force induced by 24-h TNFα exposure is due to an increase in contractile protein expression (Dogan et al., 2017; Sieck et al., 2019). Importantly, the increase in hASM force generation induced by TNFα exposure is associated with an increase in ATP consumption and tension cost (Dogan et al., 2017). This study used an NADH-linked fluorescence technique in permeabilized hASM in which the level of Ca^{2+} activation and force generation can be controlled. In previous studies, we showed that in ASM force generation is directly related to ATP hydrolysis rate (Jones et al., 1999a,b; Dogan et al., 2017). During isometric activation of hASM, ATP hydrolysis rate is initially faster and then declines with time to a sustained level even though isometric force is maintained (the "latch" state). Thus, there is a time-dependent decline in both ATP hydrolysis rate and tension cost that is likely due to cytoskeletal remodeling (Jones et al., 1999b). When actin polymerization in hASM is inhibited, force decreases while ATP hydrolysis rate increases; thereby increasing tension cost (Jones et al., 1999a,b). Normally,

tension cost of hASM is dramatically lower than skeletal muscle, but work efficiency is remarkably high (Sieck et al., 1998). Thus, the energetics of hASM are perfectly suited to sustain force at low energy cost. In hASM cells, an increase in ATP consumption is met by stimulation of ATP synthase activity (complex V) and an increase in O_2 consumption and ATP production (**Figures 1**, **3**). However, stimulation of mitochondrial O_2 consumption results in increased ROS formation that can trigger protein unfolding and an ER stress response. Thus, we propose that the pIRE1α/XBP1s ER stress pathway represents a homeostatic response directed toward reducing O_2 consumption and ROS formation in an individual mitochondrion, while increasing mitochondrial biogenesis and mitochondrial volume density to meet the increase in ATP demand. This leads to the question of how energy demand and supply are matched with continued exposure to pro-inflammatory cytokines. Sustained contractility at reduced tension cost is a hallmark of smooth muscle function, and any perturbation should be met with a homeostatic response. One possibility is that hASM cell proliferation (hyperplasia response) provides a mechanism to maintain contractility but at reduced ATP cost per cell.

ROLE OF Mfn2 IN hASM CELL PROLIFERATION

Recent evidence suggests that Mfn2 affects several pro-proliferative pathways and that dynamic mitochondrial remodeling (balance between fusion and fragmentation/fission) governs cell proliferation (Liesa et al., 2009; Antico Arciuch et al., 2012). During cell division, the number of mitochondria or therefore mitochondrial biogenesis needs to increase so each subsequent cell has a similar number of mitochondria (Antico Arciuch et al., 2012). As a result, mitochondria fuse then fragment to generate more mitochondria. Two studies in vascular smooth muscle show that Mfn2 is critical in cell division (Liesa et al., 2009; Antico Arciuch et al., 2012). Notably, the authors show that Mfn2 interacts with several pro-proliferative kinases such as extracellular signal-regulated kinase (ERK1/2) and participates in their inactivation (Liesa et al., 2009; Antico Arciuch et al., 2012). As a result, overexpression of Mfn2 in vascular smooth muscle inhibits cell proliferation (Liesa et al., 2009; Antico Arciuch et al., 2012). Importantly, ERK1/2 activation is believed to play a critical role in hASM proliferation induced by inflammatory cytokines (Lee et al., 2001; Kip et al., 2005; Yu et al., 2013; Dragon et al., 2014; Movassagh et al., 2014). While the relation between ER stress and Mfn2 is not clearly established, studies suggest that ER stress induces cell proliferation in many cell types (Vandewynckel et al., 2013; Chen et al., 2018). Whether ER stress induces hASM cell proliferation is unknown.

THERAPEUTIC APPROACHES TARGETING ER STRESS

An increase in ROS generation is likely responsible for inflammation-induced ER stress. Based on increased ROS generation associated with asthma, the benefits of antioxidant therapeutic have been explored (Heffner and Repine, 1989; Bast et al., 1991; Buhl et al., 1996; Jain and Chandel, 2013). However, one of the challenges with antioxidant treatment is specificity both in terms of ROS targeting and localization (extracellular, cytosol or mitochondrial). It is also now recognized that ROS regulate many cellular signaling cascades and have the potential to be more harmful than beneficial. An alternative therapeutic strategy of reducing ER stress in ASM is the use of chemical chaperone. Well tolerated even at high dose, chemical chaperones are effective in reducing ER stress *in vivo*. Bunezile, the US brand name for sodium phenylbutyrate or 4-phenylbutyrate (4-PBA), is currently used for patients with urea cycle disorders. Chemical chaperones such 4-PBA or tauroursodeoxycholic acid (TUDCA) have gained considerable interest as a potential therapy for a number of other diseases including but not limited to cystic fibrosis [national clinical trial (NCT)00590538 for 4-PBA and NCT00004441 for TUDCA], amyotrophic lateral sclerosis (NCT00107770 for 4-PBA and NCT03800524 for TUDCA) and some types of cancer (NCT00006019 for 4-PBA). A recent phase 1 clinical trial for TUDCA in patients with asthma has been initiated (NCT03878654). Studies in mice showed that 4-PBA attenuated airway inflammation and also reduced airway hyperreactivity in mice model of asthma further indicating a promising therapeutic role for chemical chaperones in the pathogenesis of asthma (Hoffman et al., 2013; Kim et al., 2013; Makhija et al., 2014). The effect of 4-PBA or TUDCA on ASM were not examined and it's not clear how the chemical chaperone achieved its effect, further illustrating the need to better understand how inflammation induces ER stress in hASM.

CONCLUSION AND PERSPECTIVES

Inflammation, airway hyper-contractility and proliferative remodeling are key aspects of airways diseases such as asthma. The role of inflammation-induced ER stress with downstream impact on Mfn2 and mitochondrial function is of particular interest. The ER stress pathways have been implicated in a growing number of downstream effects ranging from cell death to cell survival. Mfn2 is involved in mitochondrial tethering to the ER, mitochondrial Ca^{2+} influx, O_2 consumption, and ROS formation. Surprisingly, ER stress and Mfn2 have been largely overlooked in hASM. Mitigation of inflammation-induced ER stress in hASM may represent a novel target for therapeutic intervention.

AUTHOR CONTRIBUTIONS

PD and GS wrote the manuscript.

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