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REVIEW ARTICLE

Anisotropic mechanosensitive pathways in the diaphragm and their implications in muscular dystrophies

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Abstract The diaphragm is the "respiratory pump;" the muscle that generates pressure to allow ventilation. Diaphragm muscles play a vital function and thus are subjected to continuous mechanical loading. One of its peculiarities is the ability to generate distinct mechanical and biochemical responses depending on the direction through which the mechanical forces applied to it. Contractile forces originated from its contractile components are transmitted to other structural components of its muscle fibers and the surrounding connective tissue. The anisotropic mechanical properties of the diaphragm are translated into biochemical signals that are directionally mechanosensitive by mechanisms that appear to be unique to this muscle. Here, we reviewed the current state of knowledge on the biochemical pathways regulated by mechanical signals emphasizing their anisotropic behavior in the normal diaphragm and analyzed how they are affected in muscular dystrophies.

Keywords Muscular dystrophy · Respiratory muscles · Mechanotransduction

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Introduction

The diaphragm is the principal pressure-generating skeletal muscle that drives ventilation. Therefore, the diaphragm is constantly subjected to mechanical loading and among the skeletal muscles most affected by mechanical forces. Alteration in the mechanical properties of the diaphragm may compromise respiratory muscle function and is potentially responsible for changes in the regulation of signaling pathways that respond to the mechanical environment. Inherited muscle disorders in humans as Duchenne muscle dystrophy (DMD) or severe variants of Limb girdle muscular dystrophy (LGMD) and muscle dystrophy with myositis (*mdm*) in mice provoke mechanical dysfunction of the diaphragm. Such dysfunction may facilitate premature death either directly or by other comorbidities as pneumonia and hypercapnia (Gozal 2000).

Mechanotransduction is a ubiquitous biophysical process by which cells sense their physical environment by translating physical forces and deformations into biochemical signals such as changes in intracellular calcium concentration and/or activation of diverse mechanosensing signaling pathways. In muscle, modifications in the magnitude and duration of mechanical stimuli provoke changes in gene expression that affect metabolism and produce adaptations in muscle mass and muscle function (Tidball 2005). During ventilation the diaphragm muscle is subjected to mechanical load in both directions of the muscle plane: Along the muscle fibers and transverse to the muscle fibers. Distinct mechanosensing pathways respond depending on the direction of the mechanical stimulus. We revised the current knowledge on the mechanosensing biochemical pathways in the diaphragm with emphasis on those proven to respond anisotropically and evaluated their contribution to the onset and progression of muscular dystrophy (MD).

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The structural basis of directional mechanotransduction in the diaphragm

In skeletal muscles, the force generated by the contractile filaments must be transmitted to the skeletal elements across the surface membrane of the fibers. The most widely studied sites of tension transmission are the ends of muscle fibers where they contact either connective or epithelial tissues (Trotter 1993). The structural proteins of skeletal muscle cells can be divided in two functional distinct groups: the sarcomeric proteins and the trans-membrane associated structural proteins (see schematic in Fig. 1a). The sarcomeric proteins actin, myosin and troponin are responsible for force generation whereas other sarcomeric components as titin and nebulin are partly responsible for the longitudinal transmission of muscle force (Trinick 1991). The second group consists of the proteins that form the dystrophin complex, the α-sarcoglycan and integrin–vinculin–talin complexes, and desmin and α-actinin. These proteins are concentrated at the ends of the muscle fiber where forces are transmitted along the cell membrane and in periodic structures at the Z- and M-lines of the myofibrils called costameres that connect the sarcomeric proteins with the sarcolemma (Tidball 1991; Capetanaki et al. 2007). These arrangements suggest that the mechanical environment of the muscle fiber is three-dimensional and forces are transmitted in the transverse plane of the cell as well as at the ends of the muscle fiber, parallel to the longitudinal axis of the cell (Street 1983; Boriek et al. 1998, 2001). Anistropy is defined as the material's directional dependence of a physical property such as forcé. For example, the physical forces that affect the diaphragm are anisotropic (directionally dependent). That is the longitudinal forces along the muscle fibers of the diaphragm are distinctly different from those transmitted in the transverse direction to the long axis of the fibers. Unlike most other skeletal muscles, in vivo, the diaphragm is mechanically loaded biaxially in the direction of the muscle fibers as well as in the direction perpendicular to the fibers in the plane of the muscle sheet and the response to the mechanical load shows anisotropic behavior (Boriek et al. 2001; Margulies et al. 1994). In Fig. 1b, the analysis of the mechanical properties of normal mice hemidiaphragms and biceps femoralis by uniaxial passive mechanical loading in either the direction of the muscle fibers or transverse to the fibers provides an experimental evidence of this anisotropic behavior. The increase in tension in response to increased mechanical stretch is not affected by the direction of the applied stretch in the biceps femoris muscle whereas the diaphragm shows a higher increase in tension when the stretch is applied in the longitudinal direction of its muscles fibers. Thus, the diaphragm is more extensible in the direction along the muscle fibers (longitudinal or axial) than in the perpendicular direction to its muscle fibers (transverse).

In vitro mechanics experiments conducted in diaphragms subjected to passive biaxial loading better resemble the in vivo physiological mechanical loading of the diaphragm. The observation that transverse passive stretch increases contractile muscle force production in the diaphragm (Fig. 1c) suggests the existence of structural proteins that couple longitudinal and transverse elements of the diaphragmatic muscle (Boriek et al. 2001). Potential candidates are extra-myofilament cytoskeletal structures that are part of the costameres such as desmin, integrins, and the dystrophin-associated glycoprotein complex. The availability of mutant mice null for some of these membrane-associated structural components of the striated muscle combined with complex in vitro muscle mechanics approaches uncovered the contribution of such proteins to diaphragm muscle function. Studies in mice defective in desmin allowed uncovering that deficiency of desmin intermediate filaments results in an increased diaphragm passive transverse extensibility. In addition, diaphragm muscle exhibited decreased contractile force transmission when diaphragms are loaded biaxially (Boriek et al. 2001). Similar results were found in the dystrophin associated protein α-sarcoglycan deficient mice (Patel et al. 2003). In contrast, deficiency of α7-integrin results in decreased extensibility and contractile force production of the diaphragm muscle (Lopez et al. 2005). The common feature among the three mutant mouse models is a disruption in the coupling of the passive transverse elements and contractile longitudinal filaments. This interesting finding was uncovered through the evaluation of the effect of passive biaxial mechanical loading on contractile force generation compared to passive uniaxial longitudinal loading. The resulting increase in contractile force production under biaxial loading observed in normal mice is lost or significantly reduced in mice deficient in these costameric proteins (see Fig. 1c, d). Interestingly, a mouse model deficient in merosin, an essential extracellular protein that bridges the sarcolemma with collagen, maintains the coupling between transverse and axial elements evidenced by the increased in contractile force production in the presence of transverse passive stretch in spite of alterations in passive mechanical properties of the merosin-deficient diaphragm (Jannapureddy et al. 2003). Taken together, these results strongly suggest that costameric proteins are responsible at least in part for transmitting axial contractile forces in the transverse direction to the muscle fibers of the diaphragm.

Signaling pathways involved in diaphragm muscle mechanotransduction

The knowledge of the signaling pathways that respond to mechanical forces in the inspiratory muscle were provided by different experimental approaches mainly in vitro

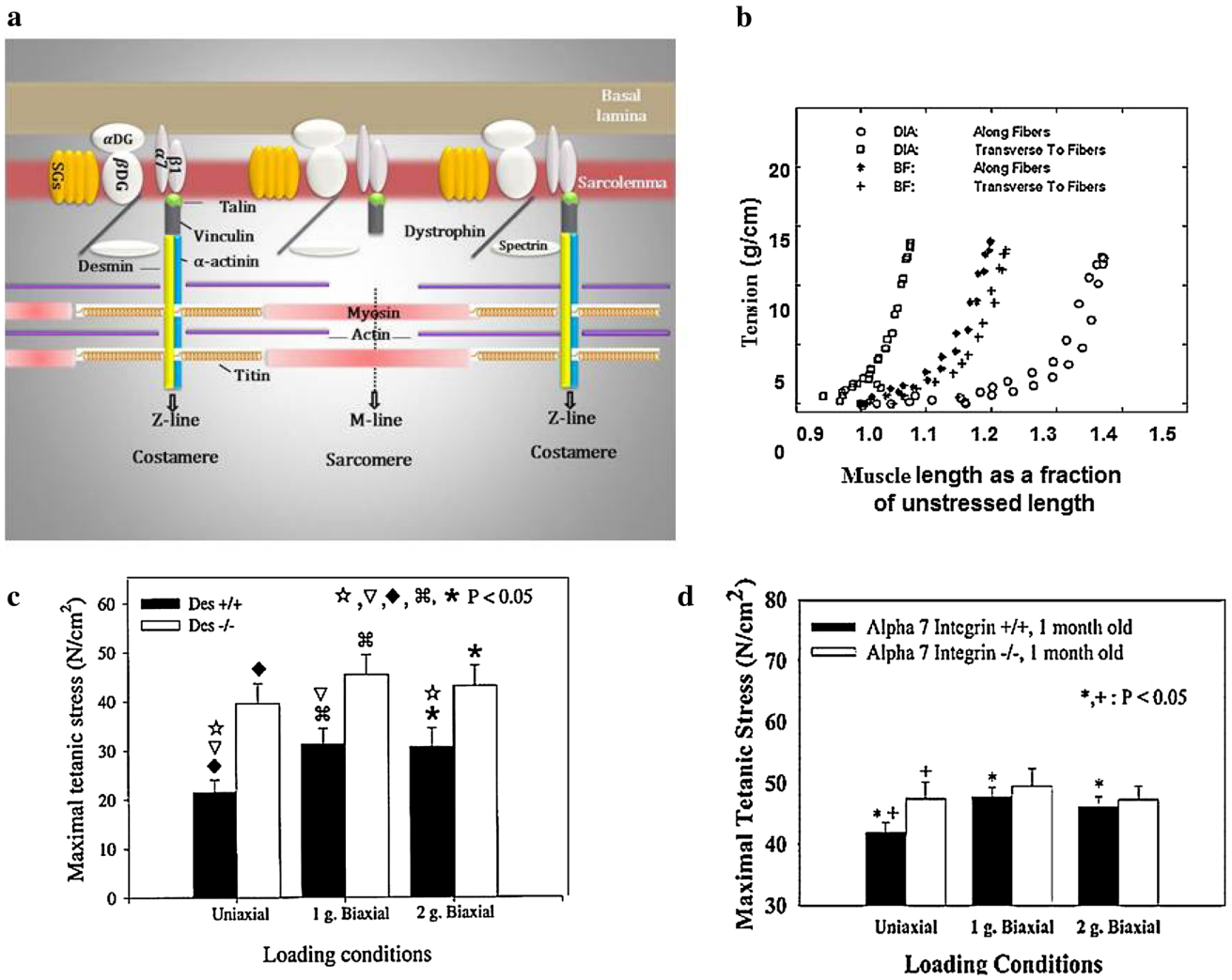

Fig. 1 Mechanical anisotropy of the diaphragm muscle. **a** Schematic view of a sarcomere of skeletal myocyte. Costameric proteins, i.e. integrins (α7, β1), and the sarcoglycan (SGs) and dystrophin glycan complex (αDG/βDG/dystrophin) aligned on the M- and Z-lines connect the contractile filaments (myosin and actin) that constitute the sarcomere with the sarcolemma and the basal lamina. Desmin and actinin are on the Z-line. Titin connects the sarcomere through the Z-disk via nebulin. **b** Muscle length–tension relationships during biaxial loading of mice diaphragm and biceps femoris muscle. Lengthening and shortening curves in the direction along the muscle fibers are represented by (open circle) and (asterisk) or by (open square) and (plus) and in the transverse direction for the diaphragm and biceps femoris respectively. Desmin decreases muscle force production. **c** Maximum tetanic stress in normal and desmin-null diaphragms under uniaxial and biaxial loading. Under both uniaxial and biaxial loadings, muscle stress production is enhanced in the absence of desmin ($p < 0.01$). Effect of biaxial loading on desmin-null diaphragm is not significant compared with that of uniaxially loaded diaphragms, whereas normal diaphragms demonstrate enhanced muscle contractile stress production in the presence of biaxial load (ANOVA: $p < 0.05$) (from Boriek et al. 2001). **d** α_7-integrin decreases muscle force production. Maximum tetanic stress in diaphragm muscle from α_7-integrin+/+ and α_7-integrin−/− mice. Muscle contractile stress increased in the α_7-integrin-null mouse diaphragmatic muscle compared to wild-type mice under conditions of longitudinal loading. However, passive transverse stretch in the direction orthogonal to the long axis of the muscle fibers has resulted in a significant increase in contractile muscle stress only in the wild-type mouse diaphragm. This strongly suggest that contractile force transmission was compromised in the α_7-integrin null mouse diaphragm. These data suggest that α_7-integrin participates in the transmission of muscle contractile force between adjacent myofibrils of the diaphragm [Previously published by Lopez et al. (2005)]

studies on normal isolated diaphragms subjected to passive mechanical stretch; (2) passive stretch of diaphragms in defective mutants and/or animal models of disease and (3) studies on animals or humans under mechanical ventilation. Each one of these approaches have provided qualitatively distinct information about the nature of mechanotransduction in the diaphragm muscle. Passive mechanical stretch experiments allowed to discriminate between pathways activated by stretch in the direction along or transverse to the muscle fibers. In addition,

results from such experiments effectively demonstrated that anisotropy of the mechanical properties of this muscle translates into anisotropy of the mechanosensing signaling pathways activated depending on the direction of the applied force. The use of defective mutants in structural muscle proteins, for which some of these are models of disease, led to uncovering the role of stretch-dependent signaling pathways in muscular dystrophy. The unloading of diaphragm muscles that occurs during mechanical ventilation brought light to the biochemical pathways that participate in diaphragm muscle weakness. In the sections below, we will review the major findings obtained by these different approaches.

The anisotropy of mechanotransduction in the diaphragm

The analysis of signaling pathways activated in response to passive stretch applied either axially or transverse to diaphragm muscle fiber direction demonstrated that two distinct pathways are responsible for the activation of ERKs and their downstream effector: the transcription factor AP-1(Fig. 2a). Though, mechanical stretch of the diaphragm in both directions of its muscle activates ERK1/2, the activation of AP-1 and ERKs is higher in the transverse direction. More strikingly, different mechanisms activate MAPK/ERK signaling in the longitudinal and transverse directions. The

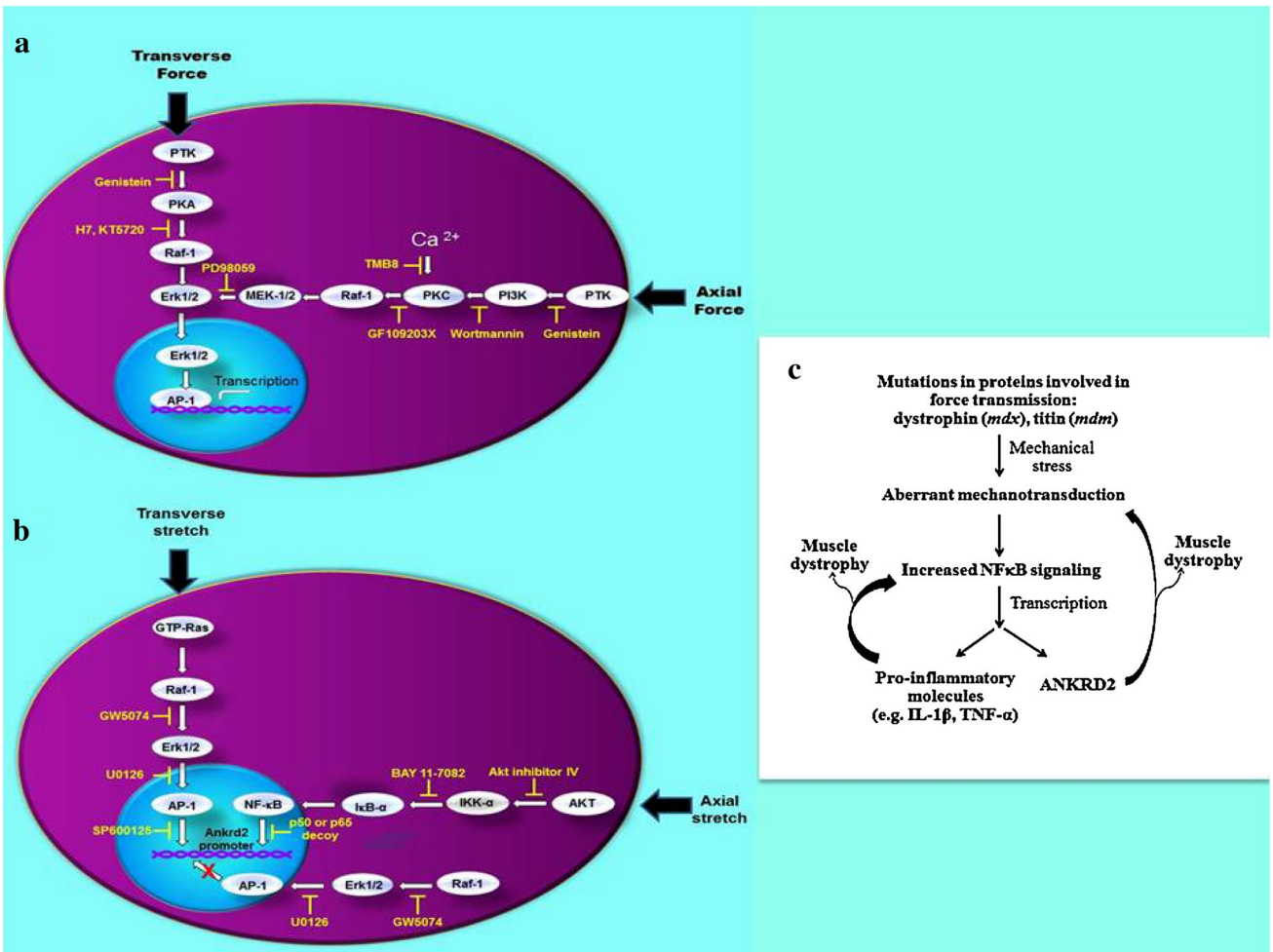

Fig. 2 Signaling pathways activated in diaphragm muscles in response to either axial or transverse mechanical stretch. **a** Mechanism of activation of ERK1/2 and AP-1 in response to mechanically loading (Kumar et al. 2002). Activation of ERK1/2 by the MEK1/2, PI3K, and PKC occurs in response to axial mechanical stress whereas PKA is involved only in response to applied transverse stress. **b** Anisotropic regulation of Ankrd2 (Mohamed et al. 2010). Longitudinal stretch up-regulates *Ankrd2* gene expression via the activation of Akt-dependent NF-κB signaling pathway. AP-1 promotes *Ankrd2* expression in the transverse direction through the activation of Raf-1/ERK1/2/AP-1 signaling pathway by GTP-Ras. **c** Proposed pathophysiologic role of the activation of NF-κB in MD in response to mechanical stress. Persistent stimulation by mechanical stress and via positive feedback loop through proinflammatory cytokines such as TNF-α and IL1-β results in overstimulation of NFκB which in turn increases *Ankrd2* expression. Inflammation and gain-of-function toxicity may ultimately result in muscular dystrophy

upstream activating mechanism in the axial direction is the activation of PKC through a PI3K mediated Ca^{2+} intracellular release. In the transverse direction, the activation of PKA mediated by protein tyrosine kinase/s is responsible for the increased MAPK/ERK signaling (Kumar et al. 2002).

The expression of the ankyrin repeat domain protein ANKRD2 is also regulated by mechanical stretch of diaphragm muscles in an anisotropic manner. ANKRD2 is a structural component of the sarcomere, which interacts with telethonin in the Z-disk (Kojic et al. 2004) and takes part of the titin/Nk2.5 signaling complex (Miller et al. 2003). It was found as a highly upregulated gene by mechanical stretch in skeletal muscles (Kemp et al. 2000). As shown in the schematic in Fig. 2b, mechanical stretch of the mouse diaphragm in the axial direction activates *Ankrd2* expression by NFκB binding to its promoter as a result of Akt and IKK stretch-dependent activation. When stretch is applied in the transverse direction, the MAPK signaling cascade and the consequent AP-1 activation are responsible for the increased expression of *Ankrd2* (Mohamed et al. 2010).

microRNAs (miRNAs) expression in the diaphragm also responds to passive stretch with different outcomes depending on the direction of the applied stretch. miRNA microarray analyses from total RNA isolated from either longitudinally stretched or transversely stretched diaphragms, identified 51 mechanosensitive miRNAs (mechanomiRs) from 700 screened miRNAs. Among 29 highly regulated mechanomiR, stretch over the transverse direction of the fiber induced 20 and longitudinal stretch induced 10, being miR382 regulated by stretch in any direction though in an opposite manner. Concomitantly, mechanical stretch also upregulated the expression of key components of the miRNA synthesis machinery Drosha, the cytoplasmic protein Dicer1, and the miRNA export protein exportin-5 (Mohamed et al. 2015).

Loss of the dystrophin affects mechanotransduction in diaphragms

Dystrophin was discovered as the protein associated with Duchenne muscular dystrophy (DMD) locus in the short arm of the human X-chromosome (Hoffman and Brown 1987). DMD is a severe genetic muscular disorder that affects 1 in 3600 newborn males (Beytía et al. 2012) and leads rapidly to muscle weakness and fatigue. It preferentially affects fast muscle fibers that are subjected to cycles of degeneration and regeneration before the slow fibers are affected (Webster et al. 1998). The murine X-linked muscular dystrophy, *mdx*, is also characterized by a complete absence of dystrophin. MD in *mdx* mice starts at the age of 3 weeks (wk) and peaks at 4–5 wk, followed by regeneration of muscle fibers of hind limb at 6–7 wk (Stedman et al. 1991; Tidball et al. 1995). In the *mdx* adult mice, there is an increased level of vinculin, talin, α-actinin and integrin as well as some elevation in the dystrophin-related protein utrophin. However, in *mdx* muscles from 2- and 3-wk old mice, before the necrotic stage, the absence of dystrophin does not disrupt the cytoskeleton (Law et al. 1994; Massa et al. 1994). Thus, changes in mechanical properties and signaling before the necrotic stage are mostly attributable to the absence of dystrophin. The *mdx* diaphragms of 3 wk-old mice showed an increase in muscle compliance compared with controls, both along and transverse to the fiber direction. Again, the change in mechanical properties of the *mdx* diaphragm muscle is accompanied by aberrant mechanotransduction. The activity of ERK1/2 and AP-1 in muscle fibers from *mdx* mice in response to equal magnitude of applied mechanical stress is significantly higher in *mdx* mice than their normal counterpart with no significant difference in the magnitude of activity of other MAP kinases. The increase in the ERK1/2 activity occurred before the onset of necrosis and is not attributable to the inflammatory response that occurs in the necrotic muscle fibers (Spencer et al. 1997). The pathway responsible for the activation of ERK1/2 was elucidated by evaluating the effect of specific pharmacologic inhibitors applied during diaphragm stretch on the activities of ERK and AP-1. An increased influx of Ca^{2+} ions, possibly through the SACs (Stretch-Activated Channels) and increased FAK1 (Focal Adhesion Kinase 1) activity were observed in the stretched *mdx* diaphragms and identified as upstream regulators of ERK signaling (Kumar et al. 2004). Increased basal and mechanical-stretch induced NFκB signaling were observed in *mdx* diaphragms of adult mice (5–6 month old). Passive mechanical stretch of the diaphragm muscle activates the IKK and the DNA binding activity of NFκB in response to stretch-induced ROS production. This enhanced mechanism in the adult *mdx* diaphragm is probably responsible for increased cytokine expression. The increased expression of inflammatory cytokines as TNF-α and IL-1β in *mdx* contributes to stretch-independent NFκB activation and synergizes the stretch dependent activation of NFκB (Kumar and Boriek 2003). Altered mechanical signaling in the *mdx* mouse (Fig. 2c) appears to be responsible at least in part for the pathological outcomes through the activation of the pro-inflammatory AP-1 and NFκB transcription factors which leads to accumulation of cytokines that potentially promote muscle wasting and enhances susceptibility to muscle injury.

The role of titin in mechanosensitive pathways in the diaphragm

Titin is a giant myofilament with multiple isoforms that range between 3 and 3.7 MDa (Bang et al. 2001). It expands through a hemi-sarcomere from the Z-disk to the M-band

(Krüger 2011). The N-terminal end of titin is anchored to the sarcomeric Z-disc via nebulin (Witt et al. 2006), α-actinin 2 (Labeit et al. 2006) and telethonin (Zou et al. 2006) and interacts with contractile filaments through its C-terminal region (Fig. 1a). The titin *mdm* mouse model resulted from a mutation that leads to aberrant splicing of four skeletal muscle specific exons that disrupts 83 amino acids of the stretch sensing domain N2A (Garvey et al. 2002). The mdm mice exhibit a clinical course similar to human DMD; they display a rapidly progressive skeletal muscle-wasting disease that ends in premature death likely due to respiratory insufficiency. The titin *mdm* mouse model is an example of a MD resulting from a mutation in a non-costameric protein in the context of an intact sarcolemma membrane. The study of passive length tension relationships, contractile force-generating capacity, and morphometric myofiber properties show that the *mdm* mouse diaphragm undergoes a progressive and deleterious structural and functional remodeling starting as early as 2 weeks of age (Lopez et al. 2008). The structural changes include a switch favoring slow myosin heavy isoforms over the fast, and decreased passive extensibility. The *mdm* diaphragm has a deformed thoracic rib cage that appears to alter the natural mechanical loading environment of the diaphragm. Such altered mechanical environment potentially contributes to an overall deficit in the diaphragm's pressure generating capacity and therefore may compromise respiratory function. Published reports suggested that the N2A ligands binding to the titin N2A region is compromised in the *mdm* mouse (Witt et al. 2004; Ono et al. 2006; Hayashi et al. 2008). Among N2A ligands, ANKRD2 is induced by stretch and highly expressed in the *mdm* diaphragm. ANKRD2 expression is controlled by stretch-induced activation of NFκB. Increased basal activation of NFκB might be responsible for increased expression of ANKRD2 in the *mdm* diaphragm (Mohamed et al. 2010). Overexpression of ANKRD2 and the transcription factor Id3 was observed in skeletal muscles and primary myoblasts obtained from *mdm* mice. These two molecules interact and contribute to the inhibition of myoblasts differentiation (Mohamed et al. 2013). The downstream effects as cytokine production and overexpression of ANKRD2 contribute to the pathophysiology of MD. Thus, in the *mdm* as in the *mdx* mice, disruption of important force-sensing cytoskeletal elements leads to aberrant activation of pro-inflammatory factors such as NFκB (Fig. 2c).

Very recently, the study on anisotropically mechanomiRs followed by pathway prediction analyses in diaphragms from WT and *mdm* mice showed an overactivation of the TGF-1/SMAD signaling pathway in diaphragms from *mdm* mouse (Mohamed et al. 2015). Two highly differentially regulated miRNAs: let7e-5p and 98-5p were upregulated in WT and dowregulated in *mdm* diaphragms. The let7e-5p targets the extracellular matrix (ECM) genes Col1a1, Col1a2, Col3a1, Col24a1, Col127a1, Itga1, Itga4, Scd1, and Thbs1. Increased expression of ECM proteins is likely to contribute to diaphragm muscle fibrosis that evolves in *mdm* mice between 2 and 6 weeks. In addition, stretch up-regulated mechanomiR-98-5p in diaphragm from WT mouse, but not in the *mdm*, was identified as a negative regulator of myoblasts differentiation (Mohamed et al. 2015). These findings provide the first experimental evidence that the dysregulation of mechanomiRs contributes, at least in part, to disease progression in MD.

The effects of mechanical unloading of diaphragm muscles during mechanical ventilation

Mechanical ventilation (MV) is a life support intervention practiced on patients with respiratory failure defined as a major abnormality in gas exchange. Accumulated evidence in the past years suggest that mechanical ventilation and the consequent diaphragm inactivity and unloading causes diaphragm dysfunction with a loss of force generative capacity as a consequence of muscle fiber atrophy and contractile dysfunction. Diaphragmatic atrophy and contractile dysfunction occurs with as little as 18 h of controlled MV (Powers et al. 2002; Shanely et al. 2002). Mechanical ventilation-induced diaphragmatic atrophy is associated with oxidative injury and increased proteolytic activity. The effects of prolonged MV has been named ventilator-induced diaphragmatic dysfunction (VIDD) (Vassilakopoulos and Petrof 2004) and has negative consequences that leads to failed or delayed weaning and co-morbidities as lung injury or pneumonia and mortality in mechanically ventilated patients.

The pathways involved in MV-induced diaphragm weakness are raising increasing attention. The advances in this field were extensively reviewed by Powers and colleagues (Powers et al. 2013) and confirmed that oxidative stress plays a major role in VIDD (Powers et al. 2004). ROS production essentially by mitochondria is an early event in VIDD that happens as soon as 6 h of MV in the rat diaphragm (Powers et al. 2004; Zergeroglu et al. 2003). Fiber muscle atrophy occurs as the result of an altered balance between protein synthesis and protein degradation which affects the content of cytoskeletal proteins. Oxidative stress is linked to proteins oxidation and the activation of several proteolytic systems as calpain, caspases, the muscle-specific ubiquitin ligases MAF-1 and MURF, the 20S and 26S proteasome and the release of the lisosomal proteases by the process defined as autophagy involved in organelles and protein digestion (Powers et al. 2013). Oxidative stress is involved in deregulation of calcium metabolism as the result of altered "leaky" ryanodine receptors which might be involved in the activation of calpain and caspases in MV-induced fiber atrophy in the diaphragm (Matecki et al. 2016). Calpains are a class of

Ca^{2+}-sensitive cysteine proteases that mediate the disassembly of the sarcomere by cleaving titin and nebulin at the site of attachment to the Z disk (Goll et al. 2003). Cytochrome C release in response to increased mitochondrial ROS results in caspase 3 activation, shown to be responsible for myonuclei loss through apoptotic mechanisms in diaphragms of mechanically ventilated rats (McClung et al. 2007). A cross-talk between calpain and caspase 3 activation during prolonged MV is suggested by the fact that inhibition of calpain blunts caspase 3 activation and vice versa, though the mechanism remains unknown (Nelson et al. 2012). The involvement of the ubiquitin–proteasome system and the lysosomal autophagy mechanism in MV-diaphragm atrophy is suggested by several findings. Increased ubiquitinated proteins and higher activity of the 20S and 26S proteasome were found in diaphragms of humans and rats (Deruisseau et al. 2005; Levine et al. 2011). In mechanically ventilated humans diaphragms, it was detected a lower content of phosphorylated Akt and the active dephosphorylated form of its substrate FOXO1 (Levine et al. 2011). The involvement of FOXO in the development of VIDD was determined by delivering a dominant negative FOXO (dnFOXO) adeno-associated virus (AAV) vector directly into the diaphragm in rats (Smuder et al. 2015). The inhibition of FOXO transcription in the diaphragm during prolonged MV protected mitochondrial function, prevented caspase-3 activation and apoptosis and reduced the release of cytochrome c. FOXO1 induces the transcription of the muscle-specific ubiquitin ligases MAF-1 and MURF, involved in the ubiquitin-dependent proteasome pathway. The expression levels of both atrophins were also found elevated in mechanically ventilated human diaphragm biopsies (Levine et al. 2011) and were repressed by the dnFOXO in mechanically ventilated rat diaphragms (Smuder et al. 2015). FOXO factors are involved in skeletal muscle atrophy by a coordinated activation of the proteosomal and the autophagic lysosomal pathways (Zhao et al. 2006). An increase in the number of autophagosomes and the expression of key autophagy proteins was detected in human diaphragms under prolonged MV suggesting autophagy plays a role in MV-induced diaphragm atrophy (Hussain et al. 2010).

A decrease of IGF-1 levels was observed in diaphragms under prolonged MV (Gayan-Ramirez et al. 2003). IGF1 and other growth factors are responsible for Akt activation; thus IGF-1 might be the upstream signal that results in lower Akt activation in VIDD. IGF-1 triggers hypertrophic signals by (1) the downregulation of FOXO factors involved in the induction of atrogenes and autophagy (2) the stimulation of protein translation and synthesis through the Akt/mTOR pathway. In mechanical ventilated rats, the decrease in Akt activation in the diaphragm results in decreased phosphorylation of p70/S6K1 and the translation repressor 4E-BP1 (McClung et al. 2008). By this means, p70/S6K1 which is responsible for the activation of protein synthesis by phosphorylation of the S6 ribosomal protein remains inactive whereas 4E-BP1 translation repressor 4E-BP1 is kept in its active form. All the negative effects of mechanical ventilation mediated by Akt/mTor and Akt/FOXO signaling were overcome by a treatment with Trolox, an inhibitor of mitochondrial ROS production (McClung et al. 2008) underscoring the role of oxidative stress in diaphragm atrophy associated to mechanical ventilation not only by promoting protein degradation but through the inhibition of protein synthesis.

Mechanotransduction in the aging diaphragm

Inspiratory dysfunction is associated to healthy human aging and results in increasing risk of respiratory complications and infections at old age (Kelley and Ferreira 2017; Elliott et al. 2016). Being the diaphragm the main inspiratory muscle, its age-associated loss in force generation capacity may explain the impaired alveolar ventilation and ability to clear the airways by forceful exhalation during coughing or sneezing.

An age-related reduction in force generating capacity was observed in diaphragms of rats and mice explained by a selective atrophy of higher force generating type IIx and/or type IIb fibers (Gosselin et al. 1994a, b; Powers et al. 1996; Criswell et al. 1997; Greising et al. 2013). This specificity of fiber atrophy resembles the one observed in diaphragm atrophy due to denervation and suggests a detrimental effect of aging on neurotrophic signaling probably due to a loss of phrenic motoneurons and/or alterations in the neuromuscular junctions (reviewed in Elliott et al. 2016).

Changes in passive mechanical properties of the aging diaphragm have been reported in rats and correlated with changes in the extracellular matrix components with increased cross-linking and higher content of intramuscular collagen (Gosselin et al. 1994). In mice diaphragms, aging causes loss of muscle compliance and reduction in stress relaxation in the direction of the muscle fibers (Pardo et al. 2008). These changes are consistent with the lack of mechanosensitivity of the IKK/NFkB and Akt/FOXO signaling pathways in diaphragms from old mice. Interestingly, basal IKK and Akt activation and FOXO binding activity in old diaphragms were comparable to the ones in young diaphragms subjected to passive stretch (Pardo et al. 2008). A similar behavior occurred in the stretch-dependent induction of SIRT1 expression. Passive stretch of young mice diaphragms triggers EGR1 binding to the Sirt1 promoter followed by an increase in SIRT1 protein and RNA levels (Pardo et al. 2011). However, no EGR1 binding to the Sirt1 promoter or increase in SIRT1 protein levels were observed in response to stretch in diaphragms from old mice, though an age-dependent increase of SIRT1 protein levels during

adulthood was observed (Pardo and Boriek 2011). In rats, old diaphragms exhibit a relative higher content of slow-twitch oxidative fibers as compares with young diaphragms (Hirofumi 2013); slow twitch fibers have a higher content of SIRT1 which may account for the age-dependent changes in this protein content (Lin et al. 2002). Strikingly, SIRT1 and PGC-1 are responsible for the fast-to slow fiber type transition (Lin et al. 2002; Handschin et al. 2007; Rasbach et al. 2010) which raises the question whether the increase in SIRT1 expression is the effect or the cause of the age-related diaphragm remodeling. SIRT1 expression in skeletal muscle was shown to trigger an anti-oxidant response, the lack of mechanoresponsiveness of Sirt1 expression in old diaphragms may give account for an impaired ability to counteract the stretch-associated increase in ROS production leading to a sustained exposure to oxidative stress (Pardo et al. 2008), particularly in fat twitch fibers. Considering that activation of oxidative metabolism and greater resistance to fatigue are the hallmark of the slow twitch type I fibers, the age-related diaphragm remodeling might be more beneficial than detrimental for normal respiratory activity. However, the age-related fiber-specific diaphragm atrophy is critical in pulmonary disease as the fast fibers type IIb and IIx, with higher force generating capacity, are crucial for the non-ventilatory expulsive behaviors required for airway clearance (Prakash and Sieck 1998).

Concluding remarks

Here, we reviewed the interesting observation that the anisotropic mechanical behavior of the diaphragm translates into mechanosensing signaling mechanisms that are sensitive to the directionality of stretch. The MAPK cascade, one of the most characterized mechanosensitive signaling pathways is activated by two different distinct mechanosensing mechanisms involving PKA by transverse stretch and PKC by longitudinal stretch (Kumar et al. 2004). The expression of ANKRD2, one of the components of the mechanosensitive titin/Nk2 signaling complex is also activated in an anisotropic way through NFκB and AP-1 by longitudinal and transverse loading, respectively (Mohamed et al. 2010).

In models of skeletal muscle dystrophy as the *mdx* and *mdm* mice, increased basal and mechanical-stretch induced NFκB signaling are the result of aberrant mechanotransduction and may contribute to the pathology of the diaphragm muscle in either of these models of MD (Kumar and Boriek 2003; Mohamed et al. 2010). Dysregulation of mechanomiRs was detected in *mdm* diaphragms before the onset of MD (Mohamed et al. 2015) and also contributes to the pathological outcome in the *mdm* diaphragm by upregulating TGFβ signaling and ECM proteins expression.

The hallmarks of the aging diaphragm are (1) a loss in force generation capacity without major impact on normal respiratory function (Prakash and Sieck 1998) due to selective atrophy of the higher force generating fiber types IIb and IIx (Gosselin et al. 1994a, b; Powers et al. 1996; Criswell et al. 1997; Greising et al. 2013); and (2) constitutive activation of mechanosensitive pathways involved in protection against oxidative stress or atrophy (Pardo et al. 2011; Pardo and Boriek 2011). All these observations suggest that an age-related remodeling of the diaphragm results in adaptive constant exposure to ROS with minor loss of function.

The diaphragms of animals and humans subjected to mechanical ventilation suffer serious impairment in contractile function and atrophy named VIDD. The pathways involved in VIDD are mediated by two major signals: increased mitochondrial ROS production (McClung et al. 2008; Matecki et al. 2016) and the consequent higher cytoplasmic Ca^{2+} resulting from leaky rhyanodine receptors. Though mechanical unloading of the diaphragm is a desired consequence of this intervention, the extent of diaphragm unloading is difficult to measure and its effects might be partially counteracted by passive mechanical stretch exerted by the ventilator. The pathways involved have been shown to be mechanosensitive in other muscles but the role played by mechanosensing molecules in VIDD requires further exploration.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest with the contents of this article.

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