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Diaphragm abnormalities in heart failure and aging: mechanisms and integration of cardiovascular and respiratory pathophysiology

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Abstract

Inspiratory function is essential for alveolar ventilation and expulsive behaviors that promote airway clearance (e.g., coughing and sneezing). Current evidence demonstrates that inspiratory dysfunction occurs during healthy aging and is accentuated by chronic heart failure (CHF). This inspiratory dysfunction contributes to key aspects of CHF and aging cardiovascular and pulmonary pathophysiology including: i) impaired airway clearance and predisposition to pneumonia; ii) inability to sustain ventilation during physical activity; iii) shallow breathing pattern that limits alveolar ventilation and gas exchange; and iv) sympathetic activation that causes cardiac arrhythmias and tissue vasoconstriction. The diaphragm is the primary inspiratory muscle, hence, its neuromuscular integrity is a main determinant of the adequacy of inspiratory function. Mechanistic work within animal and cellular models has revealed specific factors that may be responsible for diaphragm neuromuscular abnormalities in CHF and aging. These include phrenic nerve and neuromuscular junction alterations as well as intrinsic myocyte abnormalities, such as changes in the quantity and quality of contractile proteins, accelerated fiber atrophy, and shifts in fiber type distribution. CHF, aging, or CHF in the presence of aging disturbs the dynamics of circulating factors (e.g., cytokines and angiotensin II) and cell signaling involving sphingolipids, reactive oxygen species, and proteolytic pathways, thus leading to the previously listed abnormalities. Exercise-based rehabilitation combined with pharmacological therapies targeting the pathways reviewed herein hold promise to treat diaphragm abnormalities and inspiratory muscle dysfunction in CHF and aging.

Keywords

respiratory muscle; fiber atrophy; weakness; dyspnea; exercise tolerance; fatigue

1. Introduction

Inspiratory muscles are essential for ventilatory and non-ventilatory activities. Beyond being active during breathing, inspiratory muscles are recruited near-maximally during expulsive behaviors, e.g., sneezing or coughing [1–3]. Therefore, loss of inspiratory muscle function can compromise gas exchange and the health of the pulmonary system. Heart failure and

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aging cause skeletal myopathy that affects both limb and inspiratory muscles. Although inspiratory muscles are also ‘skeletal muscles’, their structural, functional, and metabolic properties, and response to stressors or inactivity are strikingly different from limb muscles [4–8]. The diaphragm is the primary inspiratory muscle and, in CHF, abnormalities of the diaphragm occur earlier or to a greater extent than in limb muscles [9–13]. Similarly, aging causes diaphragm abnormalities [14–16,1]. The combined effects of aging and CHF are unclear because experimental models typically address each condition separately. We can deduce, from clinical measures of inspiratory function, that CHF accentuates aging-induced diaphragm abnormalities. Diaphragm dysfunction will contribute to decrease quality of life as well as enhance morbidity and mortality associated with CHF or aging. Therefore, it is imperative to understand the causes of inspiratory (or diaphragm) abnormalities to develop rational pharmacological and rehabilitation strategies that improve quality of life, reduce cardiovascular and pulmonary complications, and increase longevity in CHF patients and the elderly.

2. Evidence of inspiratory dysfunction – Heart Failure and Aging

In the clinical setting, inspiratory dysfunction is documented via ‘respiratory muscle tests’ such as maximal inspiratory pressure [17]. Considering that the diaphragm is the primary inspiratory muscle, investigators assume that abnormal outcomes of respiratory muscle tests reflect diaphragm muscle dysfunction. Because clinical tests are generally voluntary and measurements are performed mainly at the mouth or nostrils, we consider that clinical tests reflect abnormalities of the ‘inspiratory system’ (phrenic motor neurons, neuromuscular junction, and muscles). Thus, we refer to clinical measures as indicators of inspiratory (dys)function in the current review.

Inspiratory dysfunction has received greater attention in CHF than healthy aging, but evidence of inspiratory dysfunction in aging predates studies in CHF [18]. Specifically, results from the initial studies that optimized the technique to measure maximal inspiratory pressure and defined reference values were some of the first indications that aging impairs inspiratory function [19]. In a cross-sectional analysis, Black & Hyatt reported that age was inversely correlated with maximal inspiratory pressure (MIP). Ever since, age has been considered an important determinant of MIP and has been used in prediction equations to define normal values in healthy subjects [17]. Although the relationship between age and MIP has been considered linear, targeted analyses of older age groups have revealed a steeper decline in MIP for subjects older than 65 years of age. Individuals in age groups averaging 25 to 65 years of age demonstrate an approximate 30% decline in MIP with age [20], whereas MIP decreases by 60 to 70% in subjects 80 years of age and older [21,22]. These observations (illustrated in Fig. 1) and measurements of transdiaphragmatic pressure [23,24] are consistent with age-induced inspiratory dysfunction. Inspiratory dysfunction in aging may reflect overall neuromuscular abnormalities due to sarcopenia. Future studies will have to define whether aging affects diaphragm and limb muscles in a similar manner. Differences are likely to exist because of the lifelong activity of the diaphragm and aging-specific changes in respiratory system mechanics.

Chronic heart failure exacerbates the impairment in inspiratory function due to age. An early study by Hammond et al. [10] demonstrated that patients with severe biventricular CHF had a ~50% decrease in MIP. Several studies followed, which focused on more homogenous groups, and showed that CHF patients have decreased MIP [25–31]. Results from volitional as well as non-volitional tests using phrenic nerve stimulation have confirmed the decrease in inspiratory (mouth or transdiaphragmatic) pressure in CHF [31–33]. The inability to generate normal inspiratory pressure is independent of the etiology of disease [34,31,10,35]. Notably, the prevalence of this inspiratory dysfunction in CHF, defined arbitrarily as MIP < 70% predicted [35,36], does appear to depend on age. In a general outpatient population of CHF patients (age 50–60 yrs and NYHA classes I to III), the prevalence of inspiratory dysfunction is 30–50% [36,35,37]. However, conservative estimates suggest that approximately 60% of CHF patients (Class II and III) with 67 ± 9 years of age have inspiratory dysfunction [38], whereas the prevalence was 70–75% for a group of older patients (75 ± 11 years of age) with acute exacerbation of heart failure [39].

An important aspect often overlooked is that the level and prevalence of inspiratory dysfunction in CHF depends on the stage of the disease. Patients with severe CHF (Class III or IV) are weaker than patients with mild CHF (Class I) [40,32,31]. This concept is illustrated in Fig 2A, showing a cross-sectional observation of progressively lower MIP in patients ranging from New York Heart Association Class I to IV. Measurements of trans-diaphragmatic pressure with magnetic phrenic nerve stimulation have also added support to this notion of concurrent worsening of diaphragm weakness and disease severity [33]. In addition to lower maximal inspiratory pressure, the diminished ability to sustain submaximal efforts also characterizes respiratory dysfunction in CHF [35,41,42]. Specifically, the time to task-failure is substantially shorter when patients perform inspiratory efforts against a submaximal pressure-threshold load (Fig. 2B, and [35]) or isocapnic hyperpnoea [43]. In summary, inspiratory dysfunction is highly prevalent in older CHF patients or those in advanced stages of the disease.

3. Relevance of inspiratory muscle dysfunction to CHF and aging (Fig. 3)

A low percentage of the maximal inspiratory pressure is utilized during quiet breathing [15,2,44]. However, decreases in maximal inspiratory muscle function, such as those seen in aging and CHF, mandate that ventilatory behaviors occur at a higher percentage of the maximal value. Loss of submaximal diaphragm function must be compensated for by increases in motor unit firing frequency and recruitment [45], which implicates diaphragm work being performed at a higher percentage of maximal capacity and a mismatch between input (phrenic nerve activity) and output (diaphragm force). The net result of diminished maximal and submaximal inspiratory function is sensation of dyspnea, compromised ability to sustain elevated ventilation during physical activity, and exercise intolerance in CHF [46,42,47].

Compensatory adaptation to loss of inspiratory muscle function also includes changes in breathing pattern. The inability to generate force, coupled with diminished lung compliance, leads to minute ventilation being achieved with low tidal volume and high breathing rate. This ‘shallow breathing’ is a common characteristic of moderate and severe CHF [48–50]. A

consequence of a shallow breathing pattern is an increase in the ratio of dead space-to-tidal volume (V_D/V_T). Elevated V_D/V_T, which in CHF reflects an inefficient breathing pattern [48], compromises alveolar ventilation and gas exchange within the lungs. Importantly, markers of impaired gas exchange during exercise have greater prognostic value than VO_2max in patients with CHF [51].

A shallow breathing pattern also elevates sympathetic activity, through the interaction of central respiratory and sympathetic neural circuits [52]. Additionally, sympathetic activity increases further because inspiratory muscle dysfunction promotes accumulation of metabolites which stimulate group IV phrenic afferent nerve fibers [53]. Stimulation of these fibers triggers reflex sympathetic activation [53,54]. Altogether, this enhanced sympathetic activity predisposes CHF patients to cardiac arrhythmias and a high risk of death [55] or vasoconstriction in limb muscles that limits whole-body exercise tolerance (relevant for CHF and aging) [53,56]. Accordingly, inspiratory muscle unloading reduces sympathetic nerve activity and increases exercise tolerance in CHF patients [57–59].

In the presence of diaphragm weakness, none of the aforementioned compensatory responses preserves cough, another expulsive behavior requiring near-maximal recruitment of inspiratory muscles [45,44]. Therefore, the inability to generate normal inspiratory pressures can impair airway clearance and predisposes individuals to pulmonary infections. Pneumonia is a common pulmonary complication with aging [60–62], and CHF patients have increased risk of hospitalization due to pneumonia [63,64]. While multiple factors will determine the higher incidence of pneumonia with aging and CHF, there is a likely contribution from the loss of inspiratory function.

The integrative observations presented in this section highlight the significance and impact of inspiratory (muscle) dysfunction in the health status and prognosis of elderly subjects and CHF patients. However, these observations are mainly of indirect nature. To establish causality, it is necessary to test the impact of therapies that specifically improve diaphragm function on clinically-relevant outcomes. Altogether, the aspects discussed above emphasize the importance of understanding the pathophysiological processes and the need for the development of new therapeutic strategies for inspiratory dysfunction. Inspiratory dysfunction in CHF does not correlate with markers of left ventricular function [65,39], is unaffected by acute decompensation of heart failure [39], and is not reversed by heart transplant [42]. These observations indicate that mechanisms beyond cardiac abnormalities *per se* are responsible for inspiratory dysfunction in CHF [12]. The findings of dysfunction using phrenic nerve stimulation are consistent with abnormalities in respiratory system mechanics, diaphragm neuromuscular transmission, excitation-contraction coupling, muscle fiber size, and the contractile apparatus.

4. Respiratory system mechanics

Chest wall compliance and lung elastic recoil decrease with aging (reviewed in [18]). The overall impact of these changes is diminished respiratory system compliance, and increased residual volume and functional residual capacity with age. The latter causes flattening of the diaphragm that diminishes its force-generating capacity. The effects of CHF on lung

volumes and mechanics are the opposite of aging. CHF decreases residual volume and functional residual capacity, e.g. [66]. The disease also increases lung stiffness [67,68]. These alterations will minimize diaphragm flattening and its impact on force generation, but will exacerbate the contribution of diminished respiratory system compliance to inspiratory dysfunction in older CHF patients. The net outcome of changes in respiratory system mechanics in CHF and aging is increased work of breathing [18,67], which is accentuated by physical activity in CHF [67].

5. Neuromuscular abnormalities

The impact of aging on diaphragm phrenic motor neuron and neuromuscular junction has been reviewed in detail recently [1]. Briefly, aging causes remodeling (enlargement and fragmentation) and loss of synaptic contact in individual neuromuscular junctions [69,70]. The associated decrease in neurotrophic factors due to denervation and neuromuscular junction abnormalities plays an important role on diaphragm dysfunction in aging [1]. The effect of CHF on diaphragm neuromuscular physiology is less clear. One study has shown that CHF causes expansion of the neuromuscular junction and enhanced expression of an embryonic-type subunit of nicotinic acetylcholine receptors [71]. Overall, the pattern of changes elicited by CHF in the diaphragm neuromuscular junction is consistent with neurodegeneration and denervation [1,72,73]. Therefore, the development of CHF in the elderly might accelerate the loss of innervation and associated neurotrophic factors and contribute to inspiratory dysfunction. Currently, it is unclear whether alterations in the neuromuscular junction precede (and cause) or are a consequence of intrinsic diaphragm muscle abnormalities in CHF.

6. Intrinsic diaphragm muscle abnormalities

6.1 - Isometric and isotonic contractile properties

Measurements of inspiratory muscle function in humans such as MIP and twitch transdiaphragmatic pressure reflect mostly isometric properties. Direct measurements of diaphragm muscle function *in vitro* and *in situ* show that isometric force normalized for cross-sectional area (‘specific force’) is depressed by 15–30% in heart failure [74–76,13,77–79] as well as aging [16,15,14,80,81]. The decrease in isometric force is seen in both twitch and maximal tetanic contractions (e.g., Fig. 4) and is independent of the etiology of disease, being evident in models of dilated and ischemic CHF [74–76,13,77–79,82,83].

Respiratory muscle tests that represent isotonic properties in humans are available [84,85], but to our knowledge these have not been applied to determine aging or CHF effects. However, animal studies have shown that isotonic contractile properties are impaired by CHF or aging. CHF decreases maximal shortening velocity by 20–30% in rodents [86,78,74,83]. Hence, peak power output, which is the product of shortening velocity and specific force, displays the most pronounced degree of diaphragm contractile dysfunction: 35–50% decrease in diaphragm peak power [83,74]. The effects of CHF on isotonic contractile properties are illustrated in Fig. 4B–C.

Aging studies have produced equivocal results for isotonic properties, but species-differences may explain this variance. Old rats and mice (24 mo old, ~75% survival) show increases or no change in maximal shortening velocity or power measured in intact diaphragm bundles [87,88]. Conversely, in hamsters, aging decreased diaphragm maximal shortening velocity and peak power [89]. Ongoing studies by our group suggest that diaphragm bundles from mice in advanced stages of aging (30 mo old, <50% survival rate) also show decreases in maximal shortening velocity and peak power [90], which are consistent with those seen in limb muscles [91,92]. Thus, impairments in diaphragm isotonic contractile properties may have a delayed onset and be more relevant in very old age. We cannot exclude, however, that discrepant results reported among species reflect differences in protocols and analytical approaches to examine isotonic properties. Ideally, the effects of age on diaphragm isotonic contractile function would have to be resolved using skinned single fibers from human samples. This approach would allow the determination of changes in isotonic contractile function specific to each MHC isoform. Yet, testing of skinned fibers from diaphragm of healthy subjects is not a trivial task because collection of biopsies has to be performed during a medically prescribed surgery in the thoracic or abdominal compartment.

Decreases in shortening velocity and power are highly relevant because diaphragm activities require muscle shortening. For instance, inspiratory time diminishes during physical activity due to higher breathing frequency, while inspiratory pressure developed during each breath increases (i.e., becomes more negative) to achieve an elevated tidal volume. Additionally, reflex responses such as coughing and sneezing elicit a very rapid and deep inspiration. Hence, declines in isotonic function will compromises breathing during moderate-to-high intensity physical activity and expulsive behaviors that demand fast and powerful diaphragm contractions. Overall, the impairments in both isometric and isotonic contractile properties in electrically-stimulated bundles *in vitro* are the first line of evidence that aging and CHF disrupt diaphragm excitation-contraction coupling, the contractile apparatus, or both. A switch in fiber type distribution may also account for the functional changes seen in intact bundles. Fiber atrophy is another important component of the inability to generate normal absolute force and power. These aspects are discussed in detail below.

6.2 - Excitation-contraction coupling

Technical challenges in isolating intact single fibers of the diaphragm have prevented extensive analysis of E-C coupling. Based on findings reported in limb muscles of old [93] and CHF animals [94,95] and patients [96], it is reasonable to speculate that aging or CHF impairs diaphragm calcium release. In diaphragm preparations, CHF slows calcium reuptake, which appears to be caused by decreases in sarcoplasmic reticulum calcium-ATPase expression [97–99]. Thus, abnormalities in E-C coupling may contribute to diaphragm dysfunction in CHF and aging.

6.3 - Contractile apparatus

Isometric and isotonic contraction dysfunction may also be explained at the level of the sarcomere. In permeabilized diaphragm single fibers, where calcium concentration is controlled externally, CHF decreases maximal specific force and Ca^{2+} sensitivity in all fiber

types [100,75]. Maximal specific force is determined by the total number of cross-bridges, the fraction of cross-bridges in the strongly bound force-generating state, and the force generated per cross-bridge [101,102]. The loss of maximal force in CHF is due to a decrease in the number of cross-bridges and force per cross bridge [103,78,79].

Modifications in myosin or thin-filament proteins may account for these effects of CHF on diaphragm single fiber contractile properties. CHF causes a proportional loss of diaphragm titin and myosin heavy chain (MHC) [75,104]. Loss of titin leads to wider myofilament lattice spacing and destabilization of the sarcomere that, respectively, lowers calcium sensitivity and maximal force [105,104,106]. The decrease in MHC content lessens the total number of available cross-bridges and contributes to diaphragm weakness [79,78,75]. Similarly, aging-induced decreases in myofibrillar protein content are associated with the specific force deficit in intact diaphragm bundles [80,14]. However, loss of MHC does not fully explain impairments in contractile function in CHF. The remaining myosin in diaphragm of CHF animals is abnormal as determined by *in vitro* motility assay showing ~20% slower sliding velocity, which occurs without a clear shift in MHC or myosin light chain isoforms [86]. CHF also slows cross-bridge kinetics in all fiber types [75]. Slowed sliding (shortening) velocity might be caused by decreases in myosin ATPase activity [107,101], which have been shown in all fiber types from limb muscle of CHF patients [108]. The sluggish cross-bridge kinetics is most likely related to a diminished rate of transition from weak to strong-binding state. Thus, post-translational myofibrillar abnormalities appear to be a major contributor to decreases in specific force, shortening velocity, and power determined in intact diaphragm bundles.

6.4 - Fiber type distribution and myofibrillar protein isoforms

Aging and CHF cause modest shifts in diaphragm fiber type composition. The diaphragm of old rats have 5% more type I fibers [109,88] and 10–15% more type IIb fibers [81,88] than young animals, which occurs due to proportional decreases in type IIa [109,88] and IIx/d fibers [81,88]. In old mice, Greising et al. [14] found no change in the percentage of type I fibers, with a shift to increased type IIa and decreased type IIx/d fibers. The effects of CHF (in animal studies) are generally the opposite of those elicited by aging, but findings are inconsistent among studies. Some groups have reported increases in type I and IIa fibers accompanied by decreases in type IIx/d and IIb fibers [110,13,76,111], while other groups have found no difference in fiber type distribution [82,112,113]. Variable results are also seen in studies with human diaphragm biopsies. Tikunov et al [114] reported higher type I and lower type II fiber percentage in patients with severe CHF undergoing heart transplant or left ventricular assist device placement. In contrast, Lindsay et al. [9] observed no difference in fiber type distribution in a similar population of patients. With limited numbers of subjects (n = 7–12 per group) and wide age ranges, it is difficult to draw conclusions based on studies in patients. However, correlational analysis of age *vs.* MHC type IIb within CHF patients suggests that older CHF patients have fewer type IIb fibers [114]. Tikunov et al have also found a shift from fast to slow myosin light chain, tropomyosin, and troponin (C, I, and T) isoforms in CHF. Aging causes a decrease in the fast myosin light chain 3 isoform in limb muscle that contributes to slow shortening velocity [115], and this may also occur in the diaphragm. Overall, MHC and thin-filament protein adaptations will

compromise diaphragm function during expulsive behaviors as fibers with slow myofibrillar protein isoforms have slower shortening velocity and lower peak power than fibers rich in fast isoforms.

6.5 - Diaphragm fiber Atrophy

Aging (sarcopenia) and CHF (cardiac cachexia) cause loss of muscle mass due to fiber atrophy. The diaphragm is highly susceptible to atrophy [5], and aging causes atrophy of type II fibers [14,69,116]. The effects of CHF on diaphragm fiber atrophy in animals seem dependent on animal model used (pressure- vs. volume overload), duration of CHF, and severity of disease. Pigs with CHF induced by supraventricular tachycardia have 20–40% lower cross-sectional area of type I, IIa, and IIB fibers [13]. In rats, CHF induced by myocardial infarction results in a 15% to 25% decrease in fiber cross-sectional area in some studies [11,117,97], but unchanged fiber cross-sectional area has been reported by our group [112] and others [75,111]. Similarly, there was no diaphragm atrophy in rats during late stages of CHF due to aortic stenosis [86]. The only study testing diaphragm atrophy in humans showed no change in fiber diameter for severe CHF (heart transplant) patients compared to controls, but participants had a wide age range (18 – 70 yrs) and were mostly males. We are currently working to define the effects of disease severity, age, and sex on diaphragm fiber atrophy in CHF. It is possible that diaphragm atrophy occurs in the early or mild-to-moderate stages of CHF that precede increased work of breathing. In the transition to severe CHF, the elevated work of breathing might restore fiber cross-sectional area to normal values. This pattern would mask fiber atrophy and the elevated catabolic state that is typical of severe CHF. Alternatively, the lack of diaphragm fiber hypertrophy with elevated work of breathing in CHF could reflect anabolic resistance [118]. Ultimately, diaphragm atrophy, when present, plays an important role in inspiratory dysfunction.

6.6 - Fatigue characteristics

Diaphragm abnormalities elicited by aging and CHF predispose the muscle to fatigue. Indeed, CHF accelerates isometric diaphragm fatigue *in situ* [77] and *in vitro* [82]. Conversely, in diaphragm of old animals, isometric fatigue resistance *in vitro* is unchanged or even increased [81,15]. It must be acknowledged that outcomes of isometric fatigue tested with standard protocols *in vitro*, in the presence of muscle weakness as occurs with aging and CHF, may not translate to the condition *in vivo*. Isometric fatigue is generally tested using matched stimulus frequency, i.e., mimicking a fixed phrenic motor neuron firing frequency and fully recruited motor units. Weakened muscles develop lower initial forces in matched-frequency protocols and, therefore, lower tension-time index that is a primary determinant of metabolic rate and fatigue *in vitro* [119]. Thus, it is common that weak muscles show attenuated rate of isometric fatigue in vitro [120–122,15,81]. However, the diaphragm force required to sustain breathing is unchanged (or even increased in CHF) in the presence of weakness. Therefore, a protocol using stimulus frequency adjusted to match initial specific force between conditions provides a better representation of the situation *in vivo.* The use of an ‘initial force-matched’ protocol reveals accelerated fatigue rate in the presence of diaphragm weakness [120]. Tests in patients show that CHF diminishes inspiratory muscle endurance, e.g., Fig 2. and refs [35,43]. Inspiratory endurance tests involve diaphragm shortening, and diaphragm function declines faster during repetitive

shortening contractions [123,124]. Thus, isotonic fatigue properties of the diaphragm would be more relevant for *in vivo* function, and such measurements are still lacking in aging or CHF.

7. Inflammatory and neuroendocrine factors

7.1 - Renin-angiotensin system

The renin-angiotensin system is hyperactive in CHF and aging. Angiotensin II signaling is a hallmark of activation of the renin-angiotensin system. CHF raises systemic levels of angiotensin II, whereas aging promotes local activation of the renin-angiotensin system without necessarily elevating circulating angiotensin II levels [125]. Importantly, angiotensin II infusion in mice causes diaphragm atrophy [126]. A likely mechanism behind this atrophy is that angiotensin II stimulates reactive oxygen species (ROS) production by NAD(P)H oxidase and mitochondria [127,128]. This angiotensin II response is relevant because independent studies have implicated ROS as causative agents in diaphragm atrophy and contractile dysfunction [83,5,129].

While the direct effects of angiotensin II on diaphragm contractile function are unknown, antagonism of angiotensin II type I receptor prevents elevation in diaphragm ROS and loss of specific force with mechanical ventilation [130]. Blockade of the renin-angiotensin system with angiotensin-converting enzyme inhibitors prevents diaphragm weakness in CHF animals and patients [26,79]; however, inspiratory dysfunction is prevalent in CHF patients receiving angiotensin-converting enzyme inhibitors [35,40]. In fact, 89% of CHF patients with inspiratory dysfunction were on angiotensin-converting enzyme inhibitors [131]. General benefits of inhibition of the renin-angiotensin system are also seen in aging [125,132], but the effects on the diaphragm are unknown.

7.2 - Cytokines

Inflammatory cytokines are elevated in CHF and aging. Tumor necrosis factor-alpha (TNFα) and interleukin 6 (IL-6) have been considered putative circulating factors that cause diaphragm abnormalities in CHF. Injection of TNFα in vivo, exposure to TNFα in vitro, or cardiac-specific overexpression of TNFα cause a 15–20% loss of diaphragm specific force [133,134,121]. The effects of this cytokine on diaphragm abnormalities are especially notable because the loss of specific force in animals with cardiac-specific overexpression of TNFα occurs in the absence of atrophy [121]. Administration of IL-6, however, does not reduce specific force, but causes atrophy in diaphragm fiber types I, IIa, IIb [135]. Thus, results from studies *in vivo* and *in vitro* suggest TNFα and IL-6 signaling as triggers of loss of specific force and atrophy, respectively, in the diaphragm with CHF. The difficulty in focusing on cytokines for systemic treatment is that, to date, a plethora of clinical trials has yielded neutral or negative results due to the complex innate immune response and modulation of cardiac function in heart failure (reviewed in ref. [136]). Thence, it is important to understand the myocyte-specific mechanisms of dysfunction to facilitate the development of diaphragm-targeted therapies. Intracellular downstream effectors of TNFα and IL-6 include sphingolipid signaling, reactive oxygen species (ROS), and activation of proteolytic pathways that are discussed below.

8. Cellular mediators of diaphragm abnormalities in CHF

8.1 - Sphingolipid signaling

Sphingolipids act as second-messengers in several pathways. The enzyme sphingomyelinase generates ceramide and is a critical component of sphingolipid signaling. Cytokines and angiotensin II activate sphingomyelinase [137–139], and CHF causes a 20% increase in the activity of the neutral isoform of sphingomyelinase [82]. Accordingly, there is an accumulation of ceramide (↑20%) in the diaphragm of CHF animals [82]. Experiments *in vitro* and *in vivo* support neutral sphingomyelinase and ceramide as mediators of diaphragm abnormalities. Recombinant sphingomyelinase activates calpain and causes atrophy in C2C12 myotubes [140,141]. In diaphragm bundles, exposure to sphingomyelinase or ceramide *in vitro* mimics the effects of CHF: decreased specific force, calcium-sensitivity, and fatigue resistance by disrupting contractile apparatus function [120,142]. A recent study has shown that neutral sphingomyelinase activation plays a causative role in diaphragm weakness induced by sepsis [140], a condition that, like CHF, is characterized by heightened cytokine levels. Therefore, inhibition of neutral sphingomyelinase may be protective against diaphragm dysfunction in CHF. Diaphragm weakness stimulated by sphingomyelinase is mediated by reactive oxygen species from NAD(P)H oxidases and mitochondria as well as activation of calpain [143,144,142,120,140].

8.2 - Reactive oxygen species, NAD(P)H oxidase, and mitochondrial abnormalities

The accumulation of ROS causes redox imbalance leading to protein oxidation that triggers diaphragm atrophy and impairs contractile function [145,146,129]. Systemic redox imbalance in CHF worsens as the disease progresses and is highest in severe stages of the disease [147,148]. This pattern parallels the progression of diaphragm weakness [32,40,31]. Importantly, CHF heightens ROS emission in the diaphragm, and despite reports of increased diaphragm antioxidant enzyme activity in CHF [149], markers of oxidation are increased by CHF in the diaphragm [86,150].

The main sources of ROS in diaphragm are NAD(P)H oxidases and mitochondria [151–153]. CHF heightens diaphragm mRNA and protein levels of Nox2 subunits of NAD(P)H oxidase. Phosphorylation of the Nox2 subunit $p47^{phox}$ is a critical step for enzyme activation and ROS production [154,153], and CHF increases phosphorylation of $p47^{phox}$ in the diaphragm [83]. Mice deficient in $p47^{phox}$, which lack Nox2 activity [155], are protected from CHF-induced increase in diaphragm ROS emission and impairments in isometric and isotonic contractile properties [83]. These observations suggest that $p47^{phox}$ is required for diaphragm abnormalities in CHF. Mitochondrial ROS emission is also elevated in CHF [112,150]. Systemic administration of mitochondria-targeted antioxidant blocks diaphragm mitochondrial ROS and normalizes diaphragm specific force in CHF [112]. Thus, mitochondrial ROS are a crucial component of the signaling pathway that culminates in diaphragm dysfunction in CHF. A plausible mechanism that reconciles findings in CHF is a cross-talk between Nox and mitochondria through ROS-induced ROS release [156,127,153,157].

In aging, mitochondria abnormalities are well-defined, and mitochondrial ROS have been implicated in aging-induced skeletal muscle dysfunction [158]. Aging increases mitochondrial DNA deletions in human diaphragm [159]. The pattern of mitochondrial DNA mutation is consistent with, and possibly a cause of, decreases in the activity of electron transport chain complexes I and IV found in the diaphragm of old rats [160]. Further evidence for a role of mitochondria in aging-induced skeletal muscle weakness comes from studies in transgenic mice. The overexpression of mitochondrial catalase in these mice prevented aging-induced increases in hydrogen peroxide emission and weakness in limb muscles [161]. The protection against contractile dysfunction conferred by mitochondrial catalase overexpression appears to be mediated through effects on the contractile apparatus and excitation-contraction coupling. An interesting factor that has emerged as a trigger of elevated skeletal muscle mitochondrial ROS in aging is denervation [158]. Fiber denervation heightens emission of mitochondrial ROS, which can affect the metabolic and contractile properties of innervated fibers surrounding the denervated one [162].

Proteins of the myofilament and excitation-contraction coupling are sensitive to ROS [163,164,161,165–167] such that exposure to exogenous ROS mimics the effects of CHF on muscle function by decreasing specific force [168,169], calcium sensitivity [167], and fatigue resistance [146,170–173]. The oxidant modification most often linked to contractile dysfunction is protein carbonylation. Protein carbonyls are increased in diaphragm homogenates in CHF [150] but not aging [174], and oxidation of MHC (CHF [86]) and RyR (aging limb muscle [93]) are associated with muscle weakness. Notably, carbonylation is an irreversible modification that, despite being a marker of redox imbalance and enhancing protein susceptibility to degradation [175], plays a lesser role in regulation of protein function. Instead, contractile function is modulated by oxidation of protein thiols and redox regulation of protein phosphorylation [176,177,166,167,102], and these are prominent candidate mechanisms of loss of diaphragm specific force and power with aging and CHF. Our research efforts are currently focused on exploring thiol oxidation as a molecular mechanism of diaphragm dysfunction in CHF and aging.

8.3 - Proteolytic pathways

Diaphragm atrophy and degradation of myofibrillar proteins requires activation of proteolytic pathways. All of the aforementioned pathways (cytokines, angiotensin II, sphingomyelinase, and ROS) activate proteolytic signaling in muscle [126,128,178,179,145]. Protein degradation is largely dependent on the ubiquitin-proteasome pathway [180]. In aging, the specific proteolytic pathways associated with diaphragm abnormalities are unclear. In CHF, markers of ubiquitin-proteasome pathway activation are elevated, and inhibition of the proteasome blunts diaphragm MHC degradation and loss of specific force [100]. These findings suggest that activation of the ubiquitin-proteasome pathway is an important component of diaphragm abnormalities in CHF. However, protein cleavage and dislodging from myofibrils precedes degradation of myofibrillar proteins by the proteasome [180]. These antecedent processes are performed at least partially by calpains [181], which are activated by calcium. CHF dysregulates calcium homeostasis in the diaphragm such that intracellular calcium concentration is elevated and

calpain activity is increased two-fold [97]. Calpain activation, which causes diaphragm weakness in sepsis and mechanical ventilation [182,183], is a plausible process that mediates diaphragm weakness in CHF, but a cause-and-effect relationship has not been established.

9. Therapeutic strategies to counteract inspiratory dysfunction

9.1 - Endurance training

Endurance exercise training promotes several cardiovascular and muscular benefits in CHF and aging [184,185], including improvements in inspiratory muscle function in CHF [186]. Endurance training prevents the loss of diaphragm specific force in animals with CHF with preserved ejection fraction or after injection of TNF-α [187,149]. However, endurance training does not prevent morphological abnormalities of the neuromuscular junction in CHF [71]. Thus, it is unclear if the improvements in diaphragm function are a direct effect of endurance training on the diaphragm or a secondary response to modulation of upstream circulating factors that trigger diaphragm abnormalities. One important aspect to consider is that endurance training can cause atrophy in diaphragm fibers in healthy young and old rats [188,189]. These findings suggest that endurance training may be detrimental to inspiratory function during expulsive behaviors, which relies on recruitment of type IIx/b fibers [44]. Therefore, we propose that endurance training needs to be combined with adjuvant therapies targeting the diaphragm and inspiratory function to help patients obtain the greatest long-term benefits of rehabilitation.

9.2 - Inspiratory (‘muscle’) resistance training

Inspiratory muscle resistance training (IMT) has been increasingly recognized as an integral component of the clinical management of CHF patients [190]. This therapy can provide additional benefits for CHF patients beyond those associated with endurance training alone [190–192]. In CHF, IMT increases maximal inspiratory pressure and endurance [35,131,193], reduces sympathetic nerve activity [194,195], heightens limb muscle blood flow [131], and prolongs time to fatigue or performance during whole-body exercise [196,191,197,190]. The technical and clinical aspects of IMT in CHF have been reviewed in detail elsewhere [190]. Importantly, CHF patients who undergo IMT pre-surgery have fewer pulmonary complications post-surgery [199]. Recent studies are emerging that suggest potential benefits of inspiratory resistance training in healthy older adults as well [84,200]. In older subjects, inspiratory muscle training increases peak inspiratory flow [84]. These findings are consistent with the notion that IMT might improve diaphragm function during expulsive behaviors and would be beneficial for airway clearance. To our knowledge, there are no specific studies showing that IMT decreases the incidence of pneumonia in the elderly or CHF patients. These extensive investigations are difficult to perform due to the requirements for a large number of patients and prolonged duration of training. Nonetheless, these are important studies that need to be done.

The cellular and molecular bases of improved inspiratory function with IMT in the human diaphragm are unknown. Inspiratory muscle training increases diaphragm thickness in CHF patients and old subjects [131,200], suggesting fiber hypertrophy similar to that seen in

healthy young rats undergoing a protocol that simulates IMT [201–203]. This approach also heightens diaphragm citrate synthase and cytochrome c oxidase activities in animals [204,205], suggesting greater mitochondrial volume density post-training. Neuromuscular adaptations are an important component of strength gains with resistance training in limb muscles. Therefore, the functional benefits of IMT in CHF and aging might result from diaphragm fiber hypertrophy along with metabolic and neuromuscular adaptations that have yet to be defined.

9.3 - Pharmacological agents

The existing knowledge of mechanisms of diaphragm weakness in CHF and aging support the use of drugs targeting proteolytic pathways or the myofilament. Bortezomib is a proteasome inhibitor that prevents protein degradation by the ubiquitin proteasome pathway. Systemic administration of bortezomib prevents loss of diaphragm MHC content and attenuates the decrease in maximal specific force in CHF rats [100]. A potential complication of ‘anti-atrophy’ agents for systemic use in CHF is an exacerbation of pathophysiological left ventricular remodeling and hypertrophy. Off-target effects on the left ventricle illustrate the need for isolating pathways and compounds specific to the diaphragm (or to skeletal muscles in general).

Other pharmaceutical agents combat the loss in diaphragmatic specific force by targeting myofibrillar proteins. In this regard, the calcium sensitizer levosimendan interacts with troponin C to increase calcium sensitivity. Exposure of diaphragm fibers (slow and fast isoforms) to levosimendan *in vitro* enhances calcium sensitivity in CHF animals [206]. Clinicians have been using levosimendan as a cardiac inotropic agent to treat acute or decompensated heart failure [207]. An off-label use of the drug could be the treatment of diaphragm dysfunction in patients with inspiratory dysfunction. New classes of calcium sensitizers have also been developed to target fast skeletal troponin C [208]. Human and animal diaphragm fibers exposed to fast troponin activators *in vitro* have increased calcium sensitivity, which translates into higher force generation within the physiological range of calcium concentrations [209,11]. *In vitro* treatment of intact diaphragm bundles from CHF rats with the fast troponin activator CK-2127107 increased submaximal diaphragm force to values equivalent to bundles from untreated control animals [11]. Diaphragm type II fibers are recruited mainly during expulsive behaviors [44,2,3]. Hence, fast troponin activators likely enhance inspiratory function during sneezing and coughing and might improve the patient’s ability to clear the airways.

Myosin activators are an alternative (or adjuvant) to troponin activators. A recent study showed that the myosin activator omecamtiv mecarbil increases calcium sensitivity of slow diaphragm fibers in healthy animals [210]. The efficacy of omecamtiv mecarbil to enhance diaphragm calcium sensitivity in pre-clinical models of CHF has not been tested. Early findings from clinical trials suggest that omecamtiv mecarbil diminishes dyspnea in CHF patients [211], and improved diaphragm function could contribute to the effects reported. Overall, inhibitors of proteolytic pathways and activators of myofibrillar protein function hold therapeutic potential for diaphragm dysfunction. Continued research on pathways

upstream of proteolysis and post-translational modification of myofibrillar proteins will help elucidate new drug targets to treat diaphragm abnormalities in CHF.

10. Summary and conclusions

Inspiratory dysfunction occurs with aging and is accentuated by CHF. Diaphragm neuromuscular and intrinsic myocyte abnormalities play a major role in the inspiratory dysfunction caused by CHF. Thus, diaphragm abnormalities contribute to key aspects of cardiovascular and pulmonary pathophysiology in CHF and aging including: i) impaired airway clearance and predisposition to pneumonia; ii) inability to sustain ventilation during physical activity; iii) shallow breathing pattern that limits alveolar ventilation and gas exchange; and iv) sympathetic activation that causes cardiac arrhythmias and tissue vasoconstriction. Loss of neurotrophic factors and activation of sphingolipid signaling, reactive oxygen species, and proteolytic pathways dictate changes in excitation-contraction coupling as well as the quantity and quality of myofibrillar proteins that lead to isometric and isotonic contractile dysfunction. Endurance and inspiratory resistance training combined with calcium sensitizing agents are current treatment options for inspiratory dysfunction, but these have yet to be optimized. The development of novel therapies will depend on research to further define receptors involved and specific cellular pathways leading to dysfunction.

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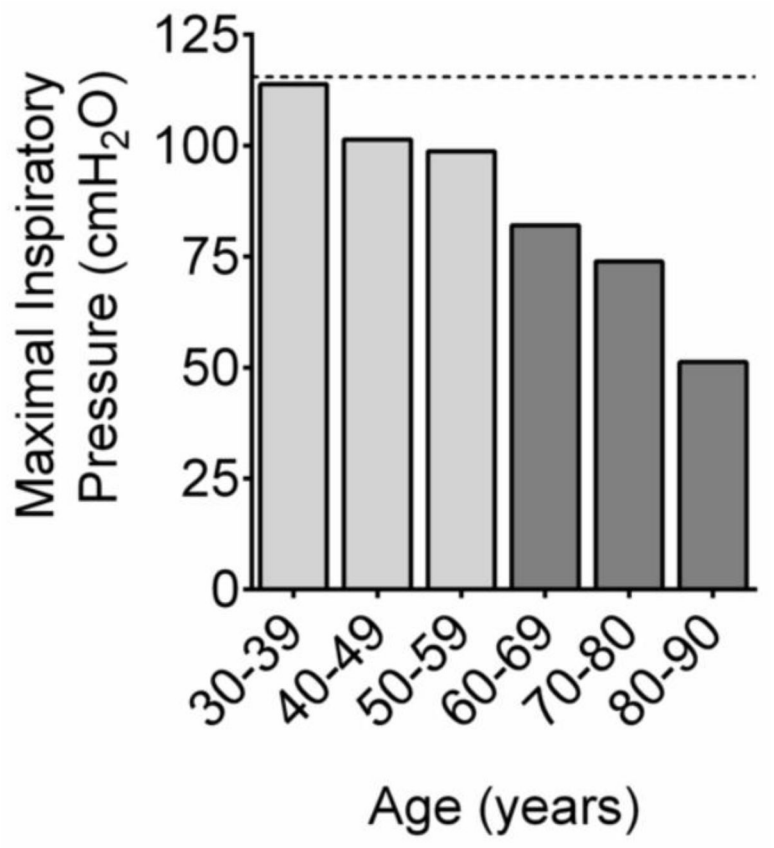

Figure 1. Decline in maximal inspiratory pressure with aging

Data are combined mean values from Neder et al. [20] and Enright et. al. [21]. Dotted line indicates mean value for subjects 20 to 29 years old.

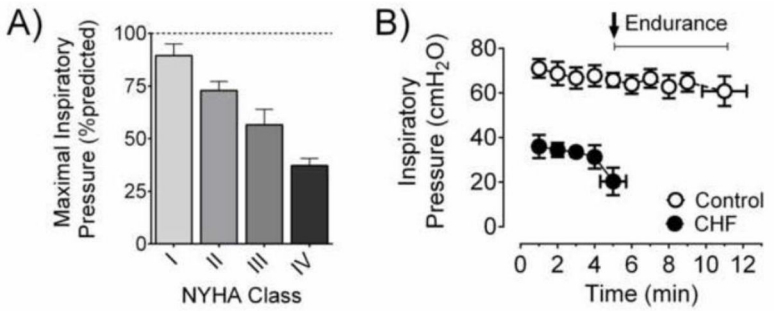

Figure 2. Inspiratory dysfunction in heart failure patients

A) Progressive decline in maximal inspiratory pressure (%predicted) in heart failure patients going from New York Heart Association (NYHA) class I to IV. Data are replotted from Filusch et al [32]. B) Heart failure patients have diminished endurance during a submaximal inspiratory load endurance test (protocol as in ref. [35]). Data shown in *panel B* were kindly provided by Dr. Gaspar Chiappa (Universidade Federal do Rio Grande do Sul, Brazil) and are from 18 CHF patients (age 64 ± 4 yrs) and 8 controls (age 62 ± 2 yrs) [131].

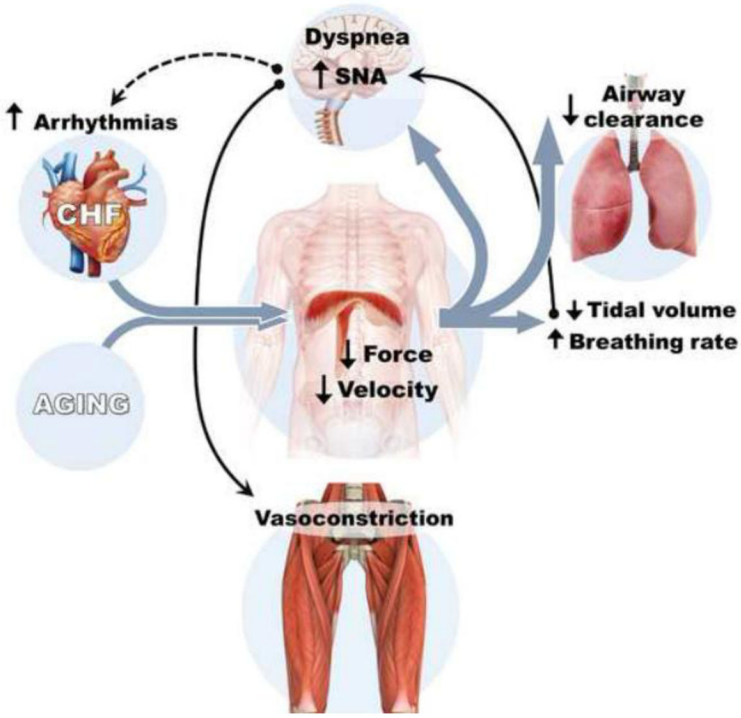

Figure 3. Relevance of diaphragm abnormalities to cardiovascular and respiratory pathophysiology in aging and heart failure

Loss of diaphragm force is caused by contractile apparatus dysfunction and fiber atrophy, whereas slower shortening velocity is determined by contractile apparatus dysfunction and fiber type shifts. These diaphragm alterations trigger cardiovascular and pulmonary pathophysiological responses. SNA, sympathetic nervous activity. Solid arrows and lines are relevant for CHF and aging, while the dotted line is relevant mainly for CHF. The model illustrated here was expanded from concepts originally developed by others [53,3,1,45].

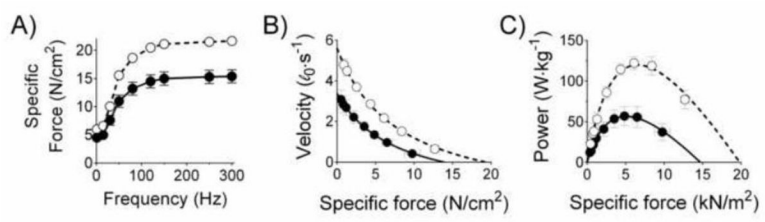

Figure 4. Heart failure causes diaphragm isometric and isotonic contractile dysfunction

Data are from intact diaphragm bundles from adult control (open circles) and CHF mice (closed circles). Specific force, force (in Newton) normalized to cross-sectional area (cm^2). Replotted from Ahn et al. [83]. The effects of aging on contractile properties are similar to those shown herein.

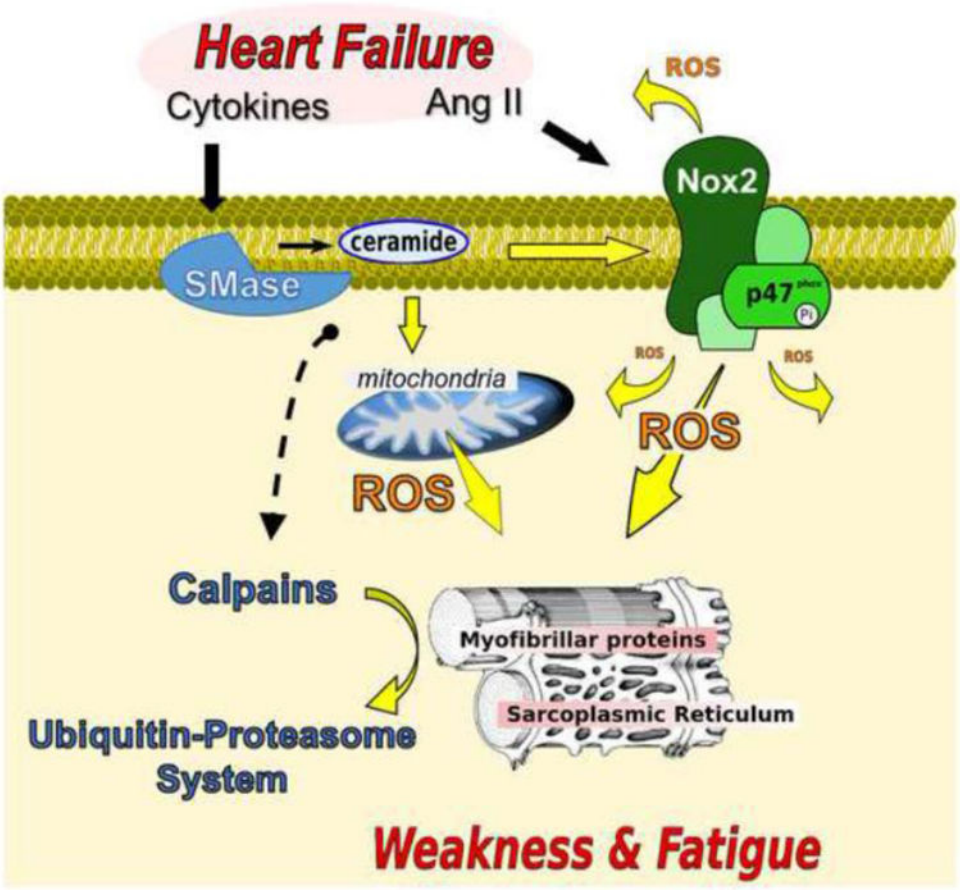

Figure 5. Circulating factors and intra-myocyte pathways leading to diaphragm abnormalities in heart failure

Angiotensin II (Ang II), sphingomyelinase (SMase), NAD(P)H oxidase 2 (Nox2), reactive oxygen species (ROS). Heart failure increases diaphragm neutral SMase activity and ceramide content [82]. SMase and ceramide cause diaphragm contractile dysfunction through ROS from mitochondria and Nox2 [142,144,143], and activation of calpain [140]. ROS play a causative role in diaphragm contractile dysfunction in heart failure [150,83,112]. Heart failure increases diaphragm calpain and proteasome activity [97,100], and proteasome inhibition blunts contractile dysfunction. Notably, ROS stimulates the ubiquitin-proteasome system [178,212].